Photosynthesis in the Light of Climate Change

Effects of Changes in the Seasonal Transitions on the Evergreen Conifer *Pinus banksiana*

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Abstract

In this PhD project different photosynthetic processes in the evergreen *Pinus banksiana*, an important conifer of the boreal forest, were assessed during the two seasonal transition periods, autumn and spring. In the experiments growth conditions under the influence of climate change were simulated. The objective of this project was to characterize physiological processes that contribute to photosynthetic adjustments and changes of boreal evergreens under future climate conditions and influence the productivity and carbon cycling of these forests.

The effect of elevated autumn air temperatures on photosynthetic capacity and energy quenching as well as the physiological mechanisms behind the plant response were investigated to evaluate how these plants will response to anticipated climate change conditions. The two main environmental signals determining the length of the growing season are photoperiod and temperature. Climate change is likely to affect temperature, but not photoperiod, which might affect the seasonal development of these trees. Using a factorial design, the effects of photoperiod and temperature on the down-regulation of photosynthetic gas exchange and the underlying mechanisms were dissected. In controlled environments control plants were grown in either warm summer conditions with 16 h photoperiod and 22 °C or conditions representing a cool autumn with 8 h photoperiod and 7 °C. To assess the impact of temperature and photoperiod on photosynthesis and energy dissipation, one set of plants was grown at 8 h photoperiod and 22 °C, representing warm autumn conditions, and another one at 16 h photoperiod and 7 °C, representing cold summer conditions. An increase in air temperature during experimental autumn conditions did not result in an increased carbon uptake by *P. banksiana* seedlings. Instead, a decrease of the photosynthetic capacity and increased rates of respiration under these conditions were observed. This was attributed, at least

in part, to an impairment of electron transport between Cyt b_6f and photosystem I. Whereas in the summer control treatment dissipation of excess energy was facilitated via zeaxanthin, dissipation in the other three treatments was predominantly dependent on aggregation of the light-harvesting-complex II and its dissociation from the photosystem II core.

Leaf reflectance spectral measurements are used in remote sensing to evaluate the physiological state of plants from leaf to ecosystem level. However, the correlation between reflectance and photosynthetic parameters might be poor under conditions where plants are cold acclimated. The recovery of photosynthesis in spring was followed in order to dissect the effect of temperature and light intensity on reflectance and photosynthetic parameters. The recovery of photosynthesis and the electron transport rate in particular was strongly temperature dependent. In contrast, the recovery of the photochemical reflectance index (PRI), estimating the amount of zeaxanthin present and used as a proxy for light use efficiency (LUE), was only dependent on light intensity. As a result, care has to be taken in order to predict LUE from PRI alone, in particular during the winter to spring transition, when the xanthophyll cycle is not yet fully active. This work underlines the importance of understanding the different quenching mechanisms under different environmental conditions.

The presented work aims to provide a better understanding on how photosynthesis is affected by climate change. It shows that it is not sufficient to look at individual factors, but that it is necessary to consider their interactive effects. The knowledge of how plants respond to changed environmental conditions is indispensable in order to accurately model the carbon balance of the world's ecosystems. An increased understanding of the physiological processes involved is necessary to move from a purely descriptive to a robust quantitative characterization of the annual global carbon cycle.

Zusammenfassung

In dieser Arbeit wurden verschiedene Photosyntheseprozesse in *Pinus banksiana*, einer wichtigen, immergrünen Kiefernart des borealen Nadelwaldes, während der zwei saisonalen Übergangsperioden im Herbst und Frühling untersucht. In den Experimenten wurden Wachstumsbedingungen unter dem Einfluss des Klimawandels simuliert. Ziel dieser Arbeit war es, die physiologischen Prozesse zu charakterisieren, die unter den künftigen Klimabedingungen zu Anpassungen und Veränderungen der Photosynthese borealer Nadelbäume beitragen und die Einfluss auf die Produktivität und den Kohlenstoffwechsel dieser Wälder haben werden.

Es wurde der Effekt von erhöhter Lufttemperatur im Herbst auf die Photosynthesekapazität und Energieverteilung, sowie die zugrunde liegenden physiologischen Mechanismen untersucht, um abzuschätzen, wie diese Pflanzen auf Klimaveränderungen reagieren werden. Tageslänge und Temperatur sind die zwei Signale, die hauptsächlich die Länge der Wachstumsperiode bestimmen. Die vorhergesagten Klimaveränderungen beeinflussen zwar die Temperatur, aber nicht die Tageslänge, was Auswirkungen auf die saisonale Entwicklung der Bäume haben könnte. Der Effekte von Temperatur und Tageslänge auf die Reduzierung des photosynthetischen Gaswechsels sowie der zugrunde liegenden physiologischen Mechanismen wurden durch ein faktorielles Experiment aufgegliedert. Kontrollpflanzen wurden in Klimakammern entweder warmen Sommerbedingungen mit 16 h Tageslänge und einer Lufttemperatur von 22 °C, oder unter Bedingungen, die kühlen Herbstbedingungen entsprechen (8 h Tageslänge und 7 °C), ausgesetzt. Um die Bedeutung von Temperatur und Tageslänge auf die Photosynthese und den Abbau von Anregungsenergie abzuschätzen, wurde ein Satz Pflanzen 8 h Tageslänge und 22 °C ausgesetzt, was warmen Herbstbedingungen entspricht, sowie ein Satz bei 16 h Tageslänge und 7 °C, entsprechend einem kühlen Sommer. Eine exper-

imentelle Erhöhung der Lufttemperatur im Herbst führte nicht zu einer Erhöhung der Kohlenstoffaufnahmen durch *P. banksiana* Setzlinge. Stattdessen wurde eine Erniedrigung der Photosynthesekapazität und eine erhöhte Respirationsrate im Vergleich mit kühlen Herbstbedingungen beobachtet. Dies wurde zumindest zum Teil einer Beeinträchtigung des Elektronentransports zwischen Cytochrom b_6f und dem Photosystem I zugeordnet. Während unter Sommerbedingungen der Abbau von überschüssiger Anregungsenergie durch Zeaxanthin vermittelt wurde, hing der Abbau in den anderen drei Bedingungen vor allem von der Aggregation des *light-harvesting*-Komplexes und dessen Dissoziierung vom Kern des Photosystem II ab.

Spektralmessungen der Reflektion von Pflanzen wird in der Fernerkundung dazu verwendet, von Blatt- zu Ökosystemebene den physiologischen Status von Pflanzen abzuschätzen. Allerdings ist nicht klar, ob die Reflektions- und Photosytheseparameter unter kälteakklimatisierten Bedingungen gut miteinander korrelieren. Die Erholung der Photosynthese im Frühjahr wurde beobachtet, um die Effekte von Temperatur und Lichtintensität auf Photosytheseparameter aufzugliedern. Die Erholung von Photosynthese und speziell der Elektronentransportrate war stark temperaturabhängig. Im Gegensatz dazu war die Erholung des *photochemical reflectance index* (PRI), der die Menge an vorhandenem Zeaxanthin abschätzt und als Indikator der Lichtnutzungseffizienz (*light use efficiency*; LUE) benutzt wird, ausschließlich lichtabhängig. Daraus folgt, dass man sehr vorsichtig beim Ableiten der LUE von PRI sein muss, besonders im Frühjahr, wenn der Xanthophyll Zyklus noch nicht vollständig aktiv ist. Diese Arbeit zeigt, wie wichtig es ist, die verschiedenen Mechanismen zum Abbau von Anregungsenergie zu kennen, die unter verschiedenen Umwelteinflüssen aktiv sind.

Die vorgelegte Arbeit zielt darauf ab, zu einem besseren Verständnis beizutragen, wie Photosynthese von Klimaveränderungen beeinflusst wird. Es wird aufgezeigt, dass eine Betrachtung einzelner Faktoren nicht ausreicht, sondern dass es notwendig ist, ihre Wechselwirkungen zu untersuchen. Das Wissen, wie Pflanzen auf geänderte Umweltbedingungen reagieren, ist unentbehrlich, um den weltweiten Kohlenstoffkreislauf korrekt zu modellieren. Hierzu ist ein besseres Verständnis der beteiligten physiologischen Prozesse notwendig, um von einer rein beschreibenden zu einer verlässlichen quantitativen Charakterisierung des globalen Kohlenstoffkreislaufs überzugehen.

Zusammenfassung der einzelnen Arbeiten

Manuskript I beschreibt als Review Artikel die bekannten Mechanismen, mit denen die Pflanze ein Gleichgewicht zwischen dem Angebot an und dem metabolischen Verbrauch von Energie herstellt. Durch Änderungen der Umweltbedingungen der Pflanze, z.B. durch eine Temperaturänderung während der Übergangsperioden, wird dieses Gleichgewicht gestört. Photosynthese selbst fungiert dabei über den Redox-Status der Komponenten der Elektronentransportkette als Sensor dieses Ungleichgewichts. Als Konsequenz einer Temperaturänderung müssen photochemische Prozesse dem metabolischen Verbrauch angepasst werden. Erstgenanntes kann z.B. durch Änderungen in der Organisation und Größe der Lichtsammel-Antennen, des funktionalen Absorptionsquerschnitts des Photosystem II und durch Anpassung der Mengenverhältnisse der Komponenten des Photosyntheseapparates geschehen. Weiterhin kann der Verbrauch durch Anpassungen des Kohlenstoffwechsels geregelt werden. Die Regulierung dieser Anpassungsreaktionen erfolgt durch ein gestaffeltes System an Prozessen, das schnelle, kurzfristige und langsamere, länger anhaltende Anpassungen ermöglicht.

Manuskript II behandelt den Effekt von erhöhter Lufttemperatur im Herbst auf die Photosynthesekapazität und Energieverteilung, sowie die zugrunde liegenden physiologischen Mechanismen an der immergrünen Bankskiefer (*Pinus banksiana*). In einem faktoriellen Experiment mit je zwei verschiedenen Tageslängen (16 h und 8 h) und Temperaturen (22 °C und 7 °C) wurde der Einfluss des erwarteten Klimawandels auf den photosynthetischen Gaswechsel untersucht. Im Vergleich zu kühlen Herbstbedingungen (8 h/7 °C; SD/LT) führte eine experimentelle Erhöhung der Lufttemperatur

im Herbst (8 h/22 °C; SD/HT) nicht zu einer Erhöhung der Kohlenstoffaufnahme durch *P. banksiana* Setzlinge. Stattdessen wurde eine Erniedrigung der Photosynthesekapazität und eine erhöhte Respirationsrate im Vergleich mit kühlen Herbstbedingungen (SD/LT) beobachtet. Gegenüber normalen Sommerbedingungen (16 h/22 °C; LD/HT) verringerte sich die Assimilations- und Respirationsrate unter SD/HT Bedingungen sogar um rund die Hälfte. Hingegen war die effektive Quantenausbeute des Photosystem II (F_v'/F_m') ausschließlich temperaturabhängig. Auch beim Abbau von überschüssiger Anregungsenergie wurden unterschiedliche Mechanismen in Pflanzen der verschiedenen Versuchsbedingungen beobachtet. Während der Abbau von Anregungsenergie unter LD/HT Bedingungen hauptsächlich durch Zeaxanthin vermittelt wurde, hing der Abbau unter SD/HT Bedingungen vor allem von der Aggregation des *light-harvesting*-Komplexes des Photosystem II ab. Unter kalten Temperaturen (LD/LT und SD/LT) kamen sowohl der Abbau von Anregungsenergie durch Zeaxanthin, als auch durch die Aggregation des *light-harvesting*-Komplexes zum Tragen. Die gesammelten Ergebnisse über den Abbau von Anregungsenergie in den Antennen und über den Elektronentransport zwischen den beiden Photosystemen wurden in einem Modell zusammengefasst.

Manuskript III greift die in Manuskript II gewonnenen Erkenntnisse auf und testet die Gültigkeit des zuvor postulierten Modells. Insbesondere wurde untersucht, an welcher Stelle der Elektronentransport beeinträchtigt war, der letztlich zur Erniedrigung der Photosynthesekapazität führte. Die Ergebnisse legen nahe, dass in SD/HT im Vergleich mit LD/HT der Elektronentransport zwischen Cytochrom b_6f-Komplex und Photosystem I beeinträchtigt war. Das folgt daraus, dass einerseits der Plastoquinon-Pool übermäßig reduziert war und andererseits eine größere Kapazität vorhanden war, den primären Elektronendonor des Photosystem I, P700, im oxidierten Zustand zu halten. Die im Modell aufgestellte Hypothese zum Abbau von überschüssiger Anregungsenergie im *light-harvesting*-Komplex konnte bestätigt werden. Obwohl der Xanthophyll Zyklus unter SD/HT Bedingungen funktionsfähig war, wurden vergleichsweise geringe Mengen Zeaxanthin nachgewiesen. Es wurde gezeigt, dass der Zeaxanthin-unabhängige Mechanismus zum Abbau von Anregungsenergie mit einer verringerten

Effizienz des Energietransfers vom *light-harvesting*-Komplex II zum Kern des Photosystem II einhergeht. Das bedeutet, dass in *P. banksiana* die Kombination von erhöhter Temperatur und kurzer Tageslänge die Struktur und Funktion des Photosyntheseapparates beeinflusste, was zu einer Reduzierung der CO_2-Assimilation führte.

Manuskript IV zeigt an einem Beispiel, wie wichtig es ist, die zuvor angesprochenen verschiedenen Mechanismen zum Abbau von Anregungsenergie zu kennen, die unter verschiedenen Umwelteinflüssen aktiv sind. Spektralmessungen der Reflektion von Pflanzen werden vermehrt in der Fernerkundung dazu verwendet, unter Sommerbedingungen von Blatt- zu Ökosystemebene den physiologischen Status von Pflanzen abzuschätzen. Da bisher noch nicht gezeigt wurde, ob die Reflektions- und Photosytheseparameter auch unter kälteakklimatisierten Bedingungen gut miteinander korrelieren, wurde die Erholung der Photosynthese im Frühjahr in einem weiteren faktoriellen Experiment beobachtet. Dadurch konnten die Effekte von Temperatur und Lichtintensität auf Reflektions- und Photosytheseparameter aufgegliedert werden. Die Erholung der Photosytheseparameter, wie z.B. der maximalen Quantenausbeute (F_v/F_m) und besonders der Elektronentransportrate, war stark temperaturabhängig. Im Gegensatz dazu war die Erholung des *photochemical reflectance index* (PRI), der die Menge an vorhandenem Zeaxanthin abschätzt und als Indikator der Lichtnutzungseffizienz (*light use efficiency*, LUE) benutzt wird, ausschließlich lichtabhängig. Da der PRI Zeaxanthin-unabhängige Mechanismen nicht berücksichtigt, folgt daraus, dass man sehr vorsichtig beim Ableiten der LUE aus PRI-Messungen sein muss. Dies gilt besonders im Frühjahr, wenn der Xanthophyll Zyklus noch nicht vollständig aktiv ist und Zeaxanthin-unabhängige Mechanismen einen wesentlichen Beitrag zum Abbau von überschüssiger Anregungsenergie leisten.

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Contents

Abbrevations

Δ**pH**	trans-thylakoid pH gradient
A	net CO_2 exchange rate (μmol photons $m^{-2} s^{-1}$)
$\mathbf{A_{350}}$, $\mathbf{A_{sat}}$	net CO_2 exchange rate at 350 μmol photons $m^{-2} s^{-1}$ and saturating light conditions
AL	actinic light
ATP	adenosine 5'-triphosphate
CET	cyclic electron transport
Chl	chlorophyll
Cyt	cytochrome
DEPS	deepoxidation state
$\mathbf{e^-}$	electron
$\mathbf{E_k}$	irradiance at which the maximum photosynthetic quantum yield balances photosynthetic capacity
ETR	electron transport rate
$\boldsymbol{F_m}$, $\boldsymbol{F_0}$	maximal, minimal fluorescence of dark-adapted leaves
$\boldsymbol{F_m'}$, $\boldsymbol{F_0'}$, $\boldsymbol{F_t}$	maximal, minimal, transient fluorescence of light-adapted leaves
$\boldsymbol{F_v}$	variable fluorescence of dark-adapted leaves ($F_v = F_m - F_0$)
$\boldsymbol{F_v/F_m}$	maximum quantum yield of PSII in dark-adapted leaves
$\boldsymbol{F_v'/F_m'}$	effective quantum yield of PSII in light-adapted leaves
Fdx	ferredoxin
FNR	ferredoxin-$NADPH^+$-oxidoreductase
FR	far red light
I	incident irradiance
LET	linear electron transport
LHC	light harvesting complex

LHCI	light harvesting complex of PSI
LHCII	light harvesting complex of PSII
LUE	light use efficiency
NADPH	nicotine adenine dinucleotide phosphate, reduced
NPQ	non-photochemical quenching (NPQ = $\frac{F_m - F_m'}{F_m'}$)
OEC	oxygen-evolving complex
P680	primary electron donor of PSII
P700	primary electron donor of PSI
PAR	photosynthetically active radiation
PC	plastocyanin
Pheo	pheophytin
PQ	plastoquinone
PQH_2	reduced plastoquinone
PRI	photochemical reflectance index
PS	photosystem
PTOX	plastid terminal oxidase
Q_A, Q_B	primary and secondary quinone
q_E	ΔpH-dependent NPQ
q_I	photoinhibitory quenching
q_N	non-photochemical quenching ($q_N = 1 - \frac{F_m' - F_0'}{F_m - F_0}$)
q_O	antenna quenching ($q_O = 1 - \frac{F_0'}{F_0}$)
q_P	fraction of open PSII reaction centers ($q_P = \frac{F_m' - F_t}{F_m' - F_0'}$)
$1 - q_P$	excitation pressure as a measure of the relative reduction state of PSII
q_T	state-transition quenching
RC	reaction center of the photosystems
R_d	respiration in the dark
ROS	reactive oxygen species
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	ribulose-1,5-bisphosphate
σ_{PSII}	effective absorption cross-section of PSII
τ^{-1}	rate at which photosynthetic electrons are consumed

Introduction

Photosynthesis is a fundamental mechanism in plant metabolism that converts light energy into biochemical usable chemical potential energy (ATP) and redox potential energy (NADPH). The photosynthetic reactions are divided into light-dependent reactions and carbon-fixation reactions. Energy generated by the light-dependent reactions is subsequently used to reduce CO_2 to form carbohydrates through the Calvin cycle of the carbon-fixation reactions. In the primary reactions of photosynthesis light energy is trapped by pigments of the into the thylakoid membrane embedded light harvesting complex (LHC) of photosystem (PS) II and PSI. Through the PSII reaction center this energy is transformed into redox potential energy, which is converted into stable reducing power in the form of NADPH. During that process a trans-thylakoid pH gradient is generated that drives ATP synthesis by chemiosmosis.

Photosynthesis and the electron transport chain

After the light energy has been absorbed by the LHC complex and passed on to PSII, linear electron transport (LET) begins at the reaction center chlorophyll (Chl), P680. The excited $P680^*$ donates an electron to pheophytin (Pheo), resulting in the formation of $P680^+$ and $Pheo^-$, a charge separation called photo-oxidation (Fig. 1). Pheophytin passes its electron on to a quinone electron acceptor, called Q_A, forming [$P680^+$ Pheo Q_A^-]. At this point, the PSII reaction center is unable to undergo another photo-oxidation event and considered to be "closed". While Q_A^- transfers the electron to plastoquinone (PQ) bound to the Q_B site on D1 polypeptide of PSII, the very strong oxidant $P680^+$ accepts an electron from water, supplied by a manganese cluster of the oxygen-evolving complex (OEC) (Minagawa and Takahashi, 2004). Now the reaction center of PSII is "open" again. The reduction of PQ, on the other hand, results in a binding of

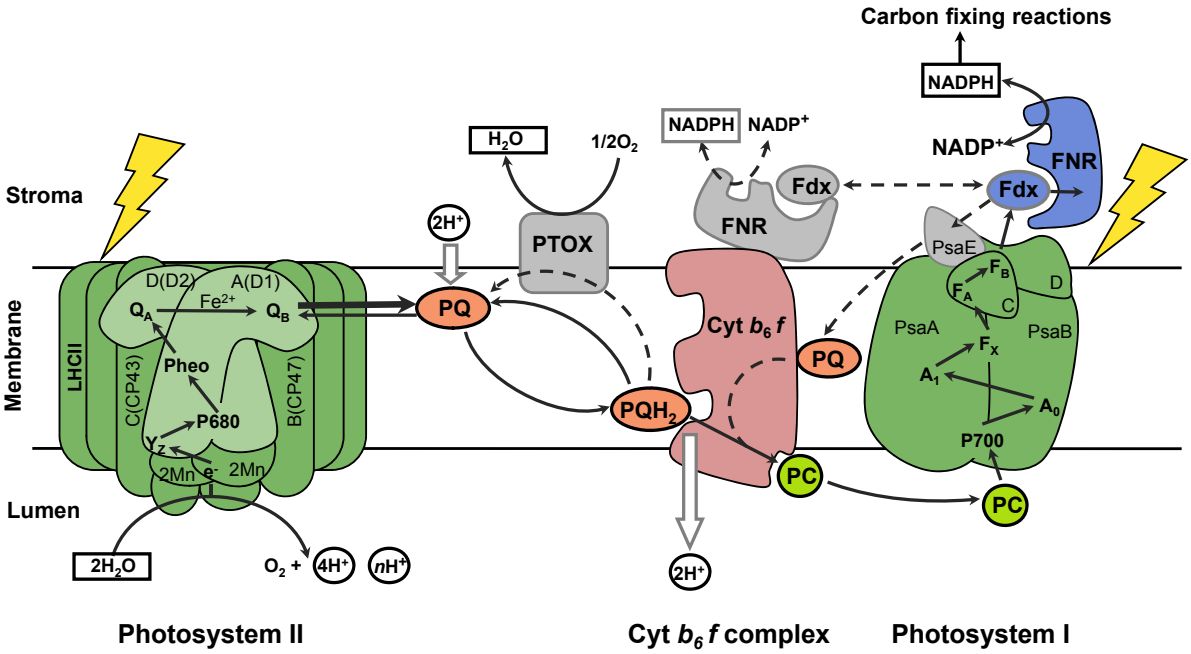

Figure 1: *Model of the thylakoid membrane showing the major polypeptides and electron transport cofactors. Solid arrows symbolize the route taken by electrons through the linear electron transport chain from the PSII RC to the carbon fixing reactions. Alternate electron routes can be taken through cyclic electron transport, donating electrons back to the PQ pool, or to directly reduce oxygen via PTOX in the chlororespiratory pathway (dashed arrows). (Modified from Wilson et al. (2006))*

two protons on the stomal side. The protons are released into the lumen once PQH_2 diffuses to and donates its electrons to the cytochrome (Cyt) b_6f complex, establishing the trans-thylakoid ΔpH and resulting in the synthesis of ATP via ATPase. The electrons are picked up from Cyt *f* by a copper-binding protein, plastocyanin (PC). PC is a small protein that is able to freely diffuse in the thylakoid luminal space. In PSI, the reaction center pigment, P700, gets excited to $P700^*$ and then oxidized to $P700^+$, similar to P680 in PSII. The primary acceptor is a Chl a and the electron is eventually passed on to ferredoxin on the stroma side of the membrane. Ferredoxin-$NADPH^+$-oxidoreductase (FNR) uses reduced ferredoxin to reduce $NADPH^+$. Finally, the electron deficiency of $P700^+$ is satisfied by withdrawing an electron from reduced PC. NADPH and ATP are used in the Calvin cycle to fix CO_2 (Wilson *et al.*, 2006).

For an optimum plant performance, the amount of energy absorbed by the photosystems and the amount of energy utilized by metabolic sinks has to be balanced, in a process called photostasis (Ensminger *et al.* 2006 I). Thus, photosynthetic processes are

highly sensitive to changes in environmental conditions. By decreasing the rates of the enzymatic reactions involved in the C, N and S reduction more strongly than the photophysical and photochemical processes involved in light absorption and energy transfer, low temperatures can exacerbate an imbalance between the source of energy and the metabolic sink (Hüner *et al.*, 1998). As a consequence, the rates of photochemical processes need to be adjusted to meet the demand of the decreased metabolic sink capacity. Photosynthesis itself acts as a sensor of the cellular energy status, through a redox sensor within the photosynthetic electron transport chain, the PQ pool (Escoubas *et al.*, 1995; Maxwell *et al.*, 1995; Allen and Nilsson, 1997; Pfannschmidt, 2003, Ensminger *et al.* 2006 I). The PQ pool transduces the electron transport signal into biochemical signals that ultimately lead to the regulation of photosynthetic genes to achieve photostasis. This regulation can result in an adjustment of the source and hence primary photosynthetic reactions and redox components, or an adjustment of the sink capacity and hence enzymes involved in the carbon metabolism (Ensminger *et al.* 2006 I). Upon a shift to low temperatures photostasis can be reached by any one or a combination of the following mechanisms: (1) by adjusting the functional cross-section of PSII; (2) by adjusting the physical size or abundance of the light harvesting complex associated with PSII; (3) by decreasing the incident irradiance and (4) by increasing the electron sink capacity by upregulation of the rate of CO_2 assimilation and the carbon metabolic pathway (Hüner *et al.*, 1998, 2003, Ensminger *et al.* 2006 I).

Photoacclimation under low temperatures - mechanisms to balance energy flow

The balance between energy source and metabolic sink can easily be disturbed by exposure to high light or low temperature, which can lead to photoinhibition, the light dependent decrease in the photosynthetic rate, and photooxidative damage. To sustain photostasis, plants have developed a wide range of mechanisms – both, rapidly reversible through an increase in thermal dissipation (non-photochemical quenching, NPQ), and long term through adjusting the stoichiometry and organization of the photosynthetic apparatus. NPQ consists of three different components that can be distinguished by their relaxation kinetics (Müller *et al.*, 2001): q_E requires the build-up of a

proton gradient and relaxes within seconds to minutes; q_T, the state-transition quenching that relaxes within tens of minutes, is associated with the separation of the major light-harvesting complex from PSII; q_I is caused by photoinhibition and shows very slow relaxation kinetics in the range of hours.

A rapid component of NPQ is a mechanism to balance energy flow through dissipation of excess energy via the xanthophyll cycle (Demmig-Adams and Adams, 1996; Demmig-Adams *et al.*, 1996a; Gilmore, 1997) (Fig. 2). The main photoprotective role of the xanthophyll cycle is to increase heat dissipation which decreases the photochemical and Chl a fluorescence efficiencies of PSII (Gilmore and Yamamoto, 1993; Gilmore *et al.*, 1998). NPQ occurs through the light-dependent conversion of violaxanthin to antheraxanthin and zeaxanthin, in combination with the PSII subunit PsbS (Li *et al.*, 2000, 2002; Savitch *et al.*, 2002; Niyogi *et al.*, 2005). Zeaxanthin and antheraxanthin are unable to pass on their excitation energy to Chl a, and thus decreasing excitation pressure by adjusting the functional cross-section of PSII (Horton *et al.*, 1999; Niyogi *et al.*, 2005). On a slightly longer timescale of minutes, state transitions can reduce

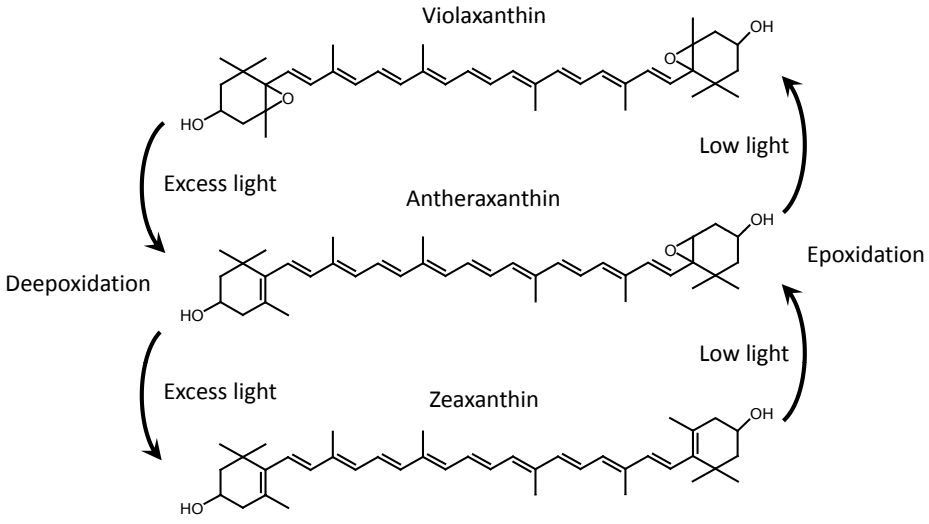

Figure 2: *Scheme of the xanthophyll cycle and its regulation by excess or limiting light. During an exposure to excess light violaxanthin is converted to antheraxanthin and further to zeaxanthin by the enzyme violaxanthin deepoxidase. In this process, a stepwise deepoxidation leads to a lengthening of the conjugated system of double bonds from nine in violaxanthin to eleven in zeaxanthin. Deepoxidation occurs within minutes. Epoxidation occurs within minutes to hours, but can take days under additional stresses (Modified from Demmig-Adams and Adams (1996)).*

excitation pressure by reducing energy-transfer efficiency to PSII through diverting energy from PSII to PSI, resulting in a balanced excitation of PSII and PSI. However, state transitions are assumed to be not of great importance for photoprotection in terrestrial plants under high light (Niyogi, 1999; Kanervo *et al.*, 2005). On a longer time scale, the components of the photosynthetic apparatus can be adjusted through the regulation of transcription and translation (Pfannschmidt *et al.*, 1999). In overwintering evergreens, a sustained form of NPQ and improved photoprotection has been observed, which is associated with the reorganization of the LHC into aggregates (Horton *et al.*, 1991; Ruban *et al.*, 1993; Ottander *et al.*, 1995; Gilmore and Ball, 2000; Krol *et al.*, 2002; Öquist and Hüner, 2003, Ensminger *et al.* 2006 I, Busch *et al.* 2007 II). Aside from NPQ, a zeaxanthin-independent way of quenching energy has been described in the reaction center of PSII, complementing antenna quenching via PsbS and the xanthophyll cycle (Ivanov *et al.*, 2001, 2006; Lee *et al.*, 2001; Sane *et al.*, 2003; Finazzi *et al.*, 2004).

In addition to antenna and RC quenching, a range of alternative electron sinks have been described. When the photon flux density is in excess of CO_2-assimilation, excess photon energy is dissipated by photorespiration, the water-water cycle, cyclic electron transport (CET) and chlororespiration (Fig. 1). Photorespiration is a metabolic pathway in which Rubisco catalyzes an oxygenase reaction, using O_2 instead of CO_2 as substrate (Wingler *et al.*, 2000). CO_2 and NH_3 are produced and ATP and reducing equivalents are consumed. Therefore photorespiration can serve as an energy sink preventing the overreduction of the photosynthetic electron transport chain, especially under stress conditions that lead to reduced rates of photosynthetic CO_2 assimilation (Streb *et al.*, 1998; Wingler *et al.*, 2000). In the water-water cycle, oxygen is photoreduced in PSI to water via O_2^- and H_2O_2. Whenever the water-water cycle operates properly for scavenging of active oxygens in chloroplasts, it also effectively dissipates excess excitation energy under environmental stress (Asada, 1999, 2006). Whereas in the linear photosynthetic electron transport the electrons are used to reduce $NADP^+$, electrons in the cyclic electron transport are fed back into the PQ pool (Munekage and Shikanai, 2005; Rumeau *et al.*, 2007) (Fig. 1). Under normal conditions, both, linear and cyclic electron transport are engaged, with LET occurring mostly in the grana and CET mostly in the stroma thylakoids (Albertsson, 2001).

The plastid terminal oxidase (PTOX) is involved in chlororespiration, by using PQH_2 to reduce O_2 (Fig. 1). In thylakoids of mature chloroplasts, chlororespiration appears to be a relatively minor pathway compared to linear photosynthetic electron transport. Even though PTOX appears to work as an electron sink, PTOX overexpression did not confer any particular resistance to PSII photoinhibition in either tobacco (Joët *et al.*, 2002) or in Arabidopsis plants (Rosso *et al.*, 2006). However, by allowing a fine-tuning of the redox state of intersystem electron carriers, PTOX may help CET to be fully operational (Joët *et al.*, 2002; Peltier and Cournac, 2002). In addition to being upregulated under stress conditons (Streb *et al.*, 2005; Quiles, 2006), PTOX has a second role and is involved in the carotenoid biosynthesis leading to β-carotene and the xanthophylls, in association with the enzyme phytoene desaturase (Wetzel *et al.*, 1994; Carol and Kuntz, 2001; Bennoun, 2002; Shahbazi *et al.*, 2007).

Cold hardening under the influence of climate change

Cold hardening in conifers is a transition of physiological processes that allow hardy plants to survive severe winter conditions. The long term adjustments of the photosynthetic apparatus and metabolism of the tree are triggered predominantly by short days and potentiated by decreasing temperatures (Weiser, 1970; Christersson, 1978; Bigras *et al.*, 2001; Beck *et al.*, 2004; Puhakainen *et al.*, 2004). An uncoupling of the two environmental signals imposes a potential imbalance on the regulation of the cold hardening process. The northern latitudes have experienced a dramatic increase in the surface air temperature, especially in the winter (ACIA, 2005; IPCC, 2007), changing one factor (temperature), while leaving the other (day length) unaffected. Several studies suggest that higher temperatures in autumn will stimulate carbon assimilation (White *et al.*, 2000; Saxe *et al.*, 2001; Churkina *et al.*, 2005), while other authors argue that any carbon gain may be offset by higher rates of respiration (Davidson and Janssens, 2006; Heimann and Reichstein, 2008; Piao *et al.*, 2008). In order to predict the terrestrial ecosystem carbon dynamics under climate change conditions, it is necessary to have a thorough understanding of the physiology of the plants. In addition, for global climate models, the actual photosynthetic activity and the 'health' status of vegetation has to be monitored on a global scale. So far, remote sensing approaches

have focussed mainly on reflectance techniques (Kogan *et al.*, 2003; Drolet *et al.*, 2005; Schaepman, 2007). A reflectance-based photosynthetic parameter, the photochemical reflectance index (PRI) was developed to provide an indication of photosynthetic light use efficiency (LUE), i.e. the amount of CO_2 assimilation per apparent photosynthetic active radiation (PAR) (Gamon *et al.*, 1990, 1992). PRI is indicative for the conversion of the xanthophyll cycle pigment violaxanthin to the deepoxidized pigments antheraxanthin and zeaxanthin (Gamon *et al.*, 1992).

Objective

The objective of this PhD project was to characterize photosynthetic processes in the boreal forest during the two transition periods, autumn and spring. Using the evergreen conifer Jack pine (*Pinus banksiana* Lamb), the effect of elevated autumn air temperatures on photosynthetic capacity and energy quenching as well as the physiological mechanisms behind the plant response were investigated to predict how these plants will response to anticipated climate change conditions. A contribution was made of how fluorescence and reflectance parameters in *P. banksiana* change over time during the recovery of photosynthesis in the spring.

Effect of elevated autumn air temperature on photosynthetic properties

Photosynthetic capacity under elevated autumn air temperature

Chlorophyll fluorescence analysis has become one of the most powerful and widely used techniques available to plant physiologists. It can give a deep functional insight into the light-dependent reactions and in how plants utilize absorbed energy. To obtain a mechanistic understanding of the carbon-fixation reactions, infrared gas analysis is commonly used. Simultaneous measurement of leaf gas exchange and modulated chlorophyll fluorescence in response to environmental conditions provide a means to determine a wide range of key biochemical and biophysical limitations on photosynthesis *in vivo* (Long and Bernacchi, 2003). One big factor impacting environmental conditions is a warming trend due to climate change (ACIA, 2005; IPCC, 2007). In the arctic, increases in temperature are observed especially in the winter, which is likely to increase the growing season by multiple days over the next decades (ACIA, 2005). This has led to the conclusion that a warming will benefit the productivity of the northern hemisphere forests (White *et al.*, 2000; Saxe *et al.*, 2001). However, northern ecosystems are particularly sensitive to increases in air temperature in autumn (Piao *et al.*, 2008). Busch *et al.* (2007 II) confirm this observation and have shown that boreal evergreen conifers might not be able to fully exploit an extended growing season. Simulating warmer air temperatures under autumn photoperiod in controlled environments, a decrease in CO_2 assimilation was reported (Busch *et al.* 2007 II). This is a consequence of an impairment of photosynthetic electron transport associated with a decreased PSII/PSI stoichiometry coupled with decreased levels of Rubisco (Busch *et al.* 2008 III).

Impairment of electron transport

What is the reason for a reduced rate of carbon assimilation? If the metabolic sink cannot keep up with the utilization of the energy that is absorbed, balancing mechanisms have to be put into place to restore photostasis. Experimentally increasing the autumn air temperature results in a situation where not all the absorbed energy ends up be-

ing used to fix CO_2 (Busch *et al.* 2007 II). The excess energy has to be either quenched non-photochemically in the reaction center or the antenna, or used in photorespiration, the water-water cycle or cyclic electron transport. However, Busch *et al.* (2008 III) have demonstrated that a reason for a decrease in CO_2 assimilation is an impairment of the electron transport between Cyt b_6f and PSI, i.e. likely a limitation in the diffusion rate of PC. In this case, it appears that a lower carbon metabolism is, at least in part, a result of an insufficient amount of reducing equivalents provided by the photosynthetic apparatus.

Dissipation of excess energy

An impairment of electron transport is not the only observed physiological change under increased autumn air temperature. A reduction in metabolic sink capacity under equal light intensity inevitably has to result in an increased capacity for dissipation of excess energy through alternate pathways (Horton and Ruban, 2005, Ensminger *et al.* 2006 I). The most proximal explanation would be an increase in NPQ through the xanthophyll cycle, as studied in detail (Demmig-Adams *et al.*, 1996b; Holt *et al.*, 2005; Niyogi *et al.*, 2005). However, under short day and increased temperatures, a dramatic decrease of the zeaxanthin and PsbS content was observed, making it unlikely that the xanthophyll cycle played a major role (Busch *et al.* 2007 II, Busch *et al.* 2008 III). Instead, increasing aggregation of LHCII (Busch *et al.* 2007 II) and its dissociation from PSII (Busch *et al.* 2008 III) support the LHCII conformation model for NPQ proposed by Horton *et al.* (2005). In this model dissipation of excess energy depends on both, the deepoxidation state of the xanthophyll cycle as well as the aggregation state of LHCII, both independently contributing to NPQ.

Modeling efforts

In carbon-cycle-climate models, the impact of climate on the carbon balance in terrestrial ecosystems is described largely by simple concepts taking into account CO_2 uptake by photosynthesis and loss by respiration. The underlying assumption adopted by researchers in the past has been that photosynthetic uptake is stimulated by both increasing CO_2 and, in boreal and temperate regions, by rising temperature, although both ef-

fects are expected to saturate at high levels of these variables (Heimann and Reichstein, 2008). Respiration, on the other hand, is assumed to be only dependent on temperature, but not affected by CO_2 concentration (Davidson and Janssens, 2006). The conclusion following from this, reflected in almost all the models, is that the biosphere is able to provide a negative feedback to rising CO_2 concentrations and temperature until the stimulating CO_2 effect is saturated (Heimann and Reichstein, 2008). However, other environmental factors or interactions between them might influence the carbon balance of the world's ecosystems. Among those are the anticipated larger climate variations and extremes (Ciais *et al.*, 2005), water and nitrogen limitations (Hyvönen *et al.*, 2007; Reichstein *et al.*, 2007) and temperature-photoperiod interactions (Busch *et al.* 2007 II). Therefore, a fundamental understanding of the processes involved and a stronger consideration of interactions between these processes is necessary to show a conclusive picture of carbon-cycle-climate system feedbacks.

Recovery of photosynthesis in spring

Thermal energy dissipation as photoprotection

The conversion of solar energy into chemical energy is the basis of life of all photosynthetic organisms. Being able to convert the light into chemical energy does not impose major problems to the plant. However, whenever sunlight is absorbed but cannot be fully utilized, the potential for damage exists. This is a problem particularly when environmental conditions limit plant growth and therefore causing photosynthetic capacity to drop while at the same time light is still absorbed by photosynthetic pigments. Evergreen conifers are exposed to prolonged environmental stress during the winter when carbon assimilation is essentially brought to a halt. Conifers have developed different photoprotective strategies to cope with this stress by thermal energy dissipation involving the interaction of chlorophyll with the carotenoid zeaxanthin (Niyogi *et al.*, 1998; Holt *et al.*, 2005; Niyogi *et al.*, 2005; Adams *et al.*, 2006). It has been shown that zeaxanthin-facilitated energy dissipation is surprisingly complex (for a recent review, see Demmig-Adams and Adams, 2006).

A flexible dissipation, which is zeaxanthin-facilitated and involves reversible F_v/F_m depressions, is ΔpH- and PsbS-dependent (Niyogi *et al.*, 1998; Li *et al.*, 2000). All plants seem to employ this PsbS/ΔpH-dependent dissipation under moderate stress, i.e. moderate levels of excess light (Demmig-Adams and Adams, 2006). Under excess light, PsbS triggers a physical rearrangement in the thylakoid membrane (Li *et al.*, 2000). PsbS might be the link between the ΔpH change that indicates the presence of excess light and a conformational change that converts violaxanthin into zeaxanthin for a flexible dissipation (Demmig-Adams and Adams, 2006). Flexible dissipation responds to changes in light intensity under favorable conditions. However, especially in evergreens, the most notable change in thermal dissipation is neither ΔpH-dependent nor rapidly reversible. Many species can maintain a high level of NPQ by maintaining a ΔpH in the dark at low temperatures (Gilmore, 1997; Demmig-Adams *et al.*, 2006). While this form of thermal dissipation can be sustained for prolonged periods in darkness and at low temperatures, it is still flexible in the sense that it can be quickly reversed upon warming of the leaves (Demmig-Adams *et al.*, 2006). Under severe and long

lasting stress conditions, a ΔpH-independent dissipation is engaged, requiring zeaxanthin (Gilmore and Ball, 2000; Demmig-Adams *et al.*, 2006). Under such conditions the xanthophyll cycle is locked in its photoprotective form and the photochemical system is primed for sustained thermal dissipation of energy (Adams *et al.*, 2006; Zarter *et al.*, 2006). This sustained photoinhibition is a major component of the protection of the photosynthetic apparatus and is indicated by a suppression of F_v/F_m (Adams and Demmig-Adams, 1994; Repo *et al.*, 2005; Ensminger *et al.*, 2008).

Recovery of photosynthesis in spring

An effective photoproctive strategy allows evergreen trees to retain much of their photosynthetic apparatus during the winter period. This enables them to quickly resume photosynthetic activity, which is assumed to be caused by warmer air temperatures (Tanja *et al.*, 2003; Sevanto *et al.*, 2006). An increase in temperature enhances the operation of biochemical reactions including repair and reorganization of the photosynthetic apparatus (Ensminger *et al.*, 2004, 2006). It has been suggested that excitation pressure on PSII and PSI, as a result of the light and temperature regime imposed by the environment, is the driving force for the onset of photosynthesis in spring in Scots pine (Ensminger *et al.*, 2004; Sveshnikov *et al.*, 2006). This will likely result in an earlier onset of photosynthesis in spring as a consequence of climate change. The start of photosynthetic activity goes along with a reduction of the sustained dissipation of excess energy and a reduction of the zeaxanthin content in the leaf (Ensminger *et al.*, 2008).

The use of remote sensing in models of gross primary production

Global estimation and monitoring of plant photosynthesis has become a very important tool to assess the impact of climate change. Modeling of the carbon cycle requires functional data of the land surface, which, on a large scale, is only possible using remote sensing. As small alterations in the terrestrial carbon balance are likely to have a significant impact on atmospheric CO_2 concentrations, there is a need for a better quantification of the changes in carbon assimilation due to climate change. Remote estimation of chlorophyll fluorescence would be one way to assess plant performance (Hilker *et al.*,

2007). However, despite recent attempts (Ananyev *et al.*, 2005; Soukupová *et al.*, 2008), it is still not feasible to remotely assess chlorophyll fluorescence parameters on a large scale. Another approach to get a direct estimation of global photosynthetic efficiency is via measuring changes in leaf spectral reflectance resulting from the deepoxidation state of the xanthophyll cycle (Gamon *et al.*, 1990). A reflectance-based photosynthetic parameter, the photochemical reflectance index (PRI), was developed to provide an indication of LUE (Gamon *et al.*, 1990, 1992). It has been shown, that the PRI reflects the deepoxidation state of the xanthophyll cycle and can be correlated with the LUE (Gamon *et al.*, 1992; Peñuelas *et al.*, 1995, 1997; Stylinski *et al.*, 2002; Trotter *et al.*, 2002). However, so far these comparisons have all been made during the growing season and it is not well understood, what the temporal and spacial requirements for observations are to accurately represent the physiological status of plant canopies (Hall *et al.*, 1995). Since different environmental conditions induce different, zeaxanthin-dependent and -independent, mechanisms of excess energy quenching (Ensminger *et al.* 2006 I, Busch *et al.* 2007 II, Busch *et al.* 2008 III), it is important to understand the causes of the quenching mechanisms. This is especially true during the transition periods of winter acclimation and spring deacclimation, given that these are the periods when major transitions in the different quenching mechanisms occur. Busch *et al.* (2008 IV) have shown that during the spring transition PRI might not correctly reflect the photosynthetic efficiency in conifers due to a temperature dependence of the photosynthetic yield, but a light dependence of the measured PRI. This suggests that a better understanding of the impact of environmental conditions on quenching mechanisms is indispensable.

Synopsis and outlook

In this thesis the effect of temperature and photoperiod on photosynthesis in the evergreen conifer *Pinus banksiana* was investigated. The focus of this study was to assess the changes in photosynthesis that might occur due to climate change during the transition periods in autumn and spring.

Photosynthetic capacity and properties of the photosynthetic apparatus were assessed during the downregulation of photosynthesis in autumn:

- An increase in air temperature during experimental autumn conditions did not result in an increased carbon uptake by *P. banksiana* seedlings.
- Instead, a decrease of the photosynthetic capacity and increased rates of respiration under these conditions were observed.
- This is, at least in part, a result of impaired electron transport between Cyt b_6f and PSI.
- Energy quenching in the antenna does not follow the well-described zeaxanthin-dependent pathway.
- Xanthophyll cycle dependent non-photochemical quenching is replaced by aggregation of LHCII and its dissociation from the PSII core.

The recovery of photosynthesis in spring was followed to dissect the effect of temperature and light intensity on photosynthetic parameters:

- The recovery of photosynthesis and photosynthetic efficiency in particular is strongly temperature dependent.
- In contrast, the recovery of the photochemical reflectance index (PRI), used as a proxy for light use efficiency (LUE), was only dependent on light intensity.
- At least during the transition period in spring care has to be taken in order to predict LUE from PRI alone.

The presented work aimed to provide a better understanding on how photosynthesis is affected by climate change. It has been shown that it is not sufficient to look at individual factors, but that it is necessary to consider their interactive effects. The knowledge of how plants respond to changed environmental conditions is indispensable in order to accurately model the carbon balance of the world's ecosystems. An increased understanding of the physiological processes involved is necessary to move from a descriptive to a quantitative characterization of the global carbon cycle.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I: Ensminger, I., **F. Busch** and N. P. A. Hüner, **2006**, Photostasis and cold acclimation: sensing low temperature through photosynthesis, *Physiologia Plantarum*, Vol. 126(1) pp 28–44
doi:10.1111/j.1399-3054.2006.00627.x

II: **Busch, F.**, N. P. A. Hüner and I. Ensminger, **2007**, Increased air temperature during simulated autumn conditions does not increase photosynthetic carbon gain but affects the dissipation of excess energy in seedlings of the evergreen Conifer Jack Pine, *Plant Physiology*, Vol. 143(3) pp 1242–1251
doi:10.1104/pp.106.092312

III: **Busch, F.**, N. P. A. Hüner and I. Ensminger, **2008**, Increased Air Temperature during Simulated Autumn Conditions Impairs Photosynthetic Electron Transport Between PSII and PSI, *Plant Physiology*, Vol. 147(1) pp 402–414
doi:10.1104/pp.108.117598

IV: **Busch, F.**, N. P. A. Hüner and I. Ensminger, **2008**, Temperature and light differentially affect light use efficiency when estimated either by chlorophyll a fluorescence or leaf spectral reflectance during the photosynthetic recovery of winter acclimated Jack pine, *Functional Plant Biology*, (submitted)

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Not included in this thesis:

Ensminger I., **Busch F.**, Caron S., Tarca A. L., MacKay J., and N. P. A. Hüner: Air temperature exerts a stronger control than photoperiod over the molecular regulation of the autumn cold hardening in needles of *Pinus banksiana*, (in preparation)

Rizzini, L., Mult, S., Hartmann, T., **Busch, F.**, Peuke, A. D., Kopriva, S., and C. Herschbach: Line specific differences in growth, photosynthesis and sulphur metabolism due to overexpression of the bacterial γ-glutamylcysteine synthetase target in plastids in grey poplar (*Populus tremula* x *P. alba*), (in preparation)

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Contributed Papers

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REVIEW

Photostasis and cold acclimation: sensing low temperature through photosynthesis

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Photosynthesis is a highly integrated and regulated process which is highly sensitive to any change in environmental conditions, because it needs to balance the light energy absorbed by the photosystems with the energy consumed by metabolic sinks of the plant. Low temperatures exacerbate an imbalance between the source of energy and the metabolic sink, thus requiring adjustments of photosynthesis to maintain the balance of energy flow. Photosynthesis itself functions as a sensor of this imbalance through the redox state of photosynthetic electron-transport components and regulates photophysical, photochemical and metabolic processes in the chloroplast. Recent progress has been made in understanding how plants sense the low temperature signal. It is clear that photosynthesis interacts with other processes during cold acclimation involving crosstalk between photosynthetic redox, cold acclimation and sugar-signalling pathways to regulate plant acclimation to low temperatures.

Figure 3: *Title page of Ensminger et al. (2006 I)*

Increased Air Temperature during Simulated Autumn Conditions Does Not Increase Photosynthetic Carbon Gain But Affects the Dissipation of Excess Energy in Seedlings of the Evergreen Conifer Jack Pine[1[OA]]

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Temperature and daylength act as environmental signals that determine the length of the growing season in boreal evergreen conifers. Climate change might affect the seasonal development of these trees, as they will experience naturally decreasing daylength during autumn, while at the same time warmer air temperature will maintain photosynthesis and respiration. We characterized the down-regulation of photosynthetic gas exchange and the mechanisms involved in the dissipation of energy in Jack pine (*Pinus banksiana*) in controlled environments during a simulated summer-autumn transition under natural conditions and conditions with altered air temperature and photoperiod. Using a factorial design, we dissected the effects of daylength and temperature. Control plants were grown at either warm summer conditions with 16-h photoperiod and 22°C or conditions representing a cool autumn with 8 h/7°C. To assess the impact of photoperiod and temperature on photosynthesis and energy dissipation, plants were also grown under either cold summer (16-h photoperiod/7°C) or warm autumn conditions (8-h photoperiod/22°C). Photosynthetic gas exchange was affected by both daylength and temperature. Assimilation and respiration rates under warm autumn conditions were only about one-half of the summer values but were similar to values obtained for cold summer and natural autumn treatments. In contrast, photosynthetic efficiency was largely determined by temperature but not by daylength. Plants of different treatments followed different strategies for dissipating excess energy. Whereas in the warm summer treatment safe dissipation of excess energy was facilitated via zeaxanthin, in all other treatments dissipation of excess energy was facilitated predominantly via increased aggregation of the light-harvesting complex of photosystem II. These differences were accompanied by a lower deepoxidation state and larger amounts of β-carotene in the warm autumn treatment as well as by changes in the abundance of thylakoid membrane proteins compared to the summer condition. We conclude that photoperiod control of dormancy in Jack pine appears to negate any potential for an increased carbon gain associated with higher temperatures during the autumn season.

Figure 4: *Title page of Busch et al. (2007 II)*

Increased Air Temperature during Simulated Autumn Conditions Impairs Photosynthetic Electron Transport between Photosystem II and Photosystem I[1[OA]]

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Figure 5: *Title page of Busch et al. (2008 III)*

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Photostasis and cold acclimation: sensing low temperature through photosynthesis

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Abstract

Photosynthesis is a highly integrated and regulated process which is highly sensitive to any change in environmental conditions, because it needs to balance the light energy absorbed by the photosystems with the energy consumed by metabolic sinks of the plant. Low temperatures exacerbate an imbalance between the source of energy and the metabolic sink, thus requiring adjustments of photosynthesis to maintain the balance of energy flow. Photosynthesis itself functions as a sensor of this imbalance through the redox state of photosynthetic electron-transport components and regulates photophysical, photochemical and metabolic processes in the chloroplast. Recent progress has been made in understanding how plants sense the low temperature signal. It is clear that photosynthesis interacts with other processes during cold acclimation involving crosstalk between photosynthetic redox, cold acclimation and sugar-signalling pathways to regulate plant acclimation to low temperatures.

Abbreviations — 1-q_P, excitation pressure as a measure of the relative reduction state of PSII; ABA, abscisic acid; Chl, chlorophyll; Cytf, protein of the cytochrome b_6f-complex; *COR*, coldregulated genes;

Introduction

Photosynthesis is the principal mechanism to transform light energy into biochemical usable chemical potential energy (ATP) and redox potential energy (NADPH) by components of the chloroplast thylakoid membrane. NADPH and ATP are then transferred to metabolic electron sinks. The main metabolic sink occurs via reduction of CO_2 through the Calvin cycle to form carbohydrates within the stroma of the chloroplast. The primary reactions of photosynthesis are temperature independent and catalysed by photosystem (PS) I and PSII to trap light energy and transform it into redox potential energy through a combination of extremely rapid ($10^{-15} - 10^{-12}$ s) photophysical and photochemical processes leading to charge separation. In contrast, much slower ($10^{-3} - 10^{0}$ s), temperature-dependent, biochemical reactions convert this redox potential energy to stable reducing power in the form of NADPH. Thereby a *trans*-thylakoid ΔpH is established by the oxygen-evolving complex and the plastoquinone (PQ) pool of the intersystem electron transport to synthesize ATP by chemiosmosis. ATP and NADPH are then consumed in the reduction of CO_2 to triose phosphate and the continuous regeneration of ribulose-1,5-bisphosphate (RuBP) on a time scale of seconds. The energy represented by the fixed carbon is then used through maintenance respiration to maintain cellular homeostasis and growth respiration for cell division and expansion on a time scale of minutes to hours. Although C reduction is considered photosynthetic, it is also important to appreciate that N and S assimilation are also photosynthetic because of their dependence on photosynthetically generated redox and

D1, see psbA; DCMU, 3-(3,4-dichlorophenyl)- 1, 1-dimethylurea; E_k, irradiance at which the maximum photosynthetic quantum yield balances photosynthetic capacity; F_v/F_m, efficiency of open PSII units; LHC, light-harvesting complex; LHCII, protein of the light-harvesting complex of photosystem II; Lhcb1 and Lhcb2, light-harvesting chlorophyll binding proteins of LHCII; NPQ, non-photochemical quenching; OEC, oxygen-evolving complex; PC, plastocyanine; PG, phosphatidylglycerol; Pi, inorganic phosphate; PPFD, photosynthetic photon flux density (μmol photons $m^{-2}\,s^{-1}$); PQ, plastoquinone; PQA, plastoquinone A; PQH_2, reduced plastoquinone; PSI or PSII, photosystem I or photosystem II, respectively; PsbA (or D1) reaction centre protein of photosystem II; PsbS, protein involved in non-photochemical quenching; Q_A or Q_B primary or secondary quinone, respectively; q_E, rapidly relaxing energy-dependent component of NPQ; RC, reaction centre of the photosystems; RuBP, ribulose-1,5-bisphosphate; SPS, sucrosephosphate-synthase; σ_{PSII}, effective absorption cross-section of PSII; τ^{-1}, rate at which photosynthetic electrons are consumed.

chemical potential energy (Paul and Foyer, 2001). Optimum plant performance requires a balance in the rates of source versus sink processes. Low temperatures, however, can inhibit electron transport by increasing membrane viscosity through alterations in the biophysical properties of thylakoid lipids and decrease the rates of the enzymatic reactions involved in C, N and S reduction more strongly than they inhibit photophysical and photochemical processes involved in light absorption, energy transfer and transformation (Hüner *et al.*, 1998). On the whole plant level, low temperatures affect the rate of growth through an inhibition of the uptake of water and nutrients. As a consequence of low temperatures, the rates of photochemical processes need to adjust to the decreased metabolic sink capacity for the consumption of photosynthates. Thus, the effects of low temperatures on photosynthesis and carbon gain eventually determine productivity, growth and distribution of plants on various spatial scales (Ball *et al.*, 1991; Farage and Long, 1991; Slot *et al.*, 2005). Cold tolerant species are able to acclimate to low temperatures. Mechanisms to overcome the constraints of short-term and long-term exposure to low temperature include changes in energy absorption and photochemical transformation through energy partitioning and concomitant changes in chloroplastic carbon metabolism, allocation and partitioning. Thus, the mechanisms involved in photosynthetic acclimation to low temperature range from modifications within the thylakoid membrane system, affecting photosynthetic electron transport (Hüner *et al.*, 1998, 2003) to post-transcriptional activation and increased expression of enzymes for sucrose synthesis, changed expression of Calvin cycle enzymes, changes in leaf protein content, as well as the signals that trigger these processes (Stitt and Hurry, 2002).

Photosynthesis reflects not only short-term responses of the chloroplast to the prevailing environmental conditions but also the physiological and developmental state of the whole organism. Cellular redox poise is one element in the puzzle which is linked, in the short term at least, to the accumulation of sugars at low temperatures. However, long-term acclimation of photosynthesis to low temperature in many plants also depends on development under low temperature. In this review, we discuss how plants adjust photosynthesis to low temperatures using Key cellular features: source activity as reflected by the redox state of the chloroplast and sink strength as reflected in

metabolic activity. In addition to the roles of source activity and sink strength in energy conversion, we suggest that both also act as global sensors of environmental change and provide the signalling required to alter specific gene expression. Large-scale genetic analysis now reveals a complex, new picture of signalling pathways involved in the process of low temperature acclimation of photosynthesis and the network that actually controls photosynthesis. These data indicate a spatial integration of signals derived from the redox state of the chloroplast and those originating from the sugar status of the cell. Finally, we discuss the concept of 'time-nested' processes (Falkowski and Chen, 2003) to address the issue of temporal integration of these signals from sources and sinks to regulate cellular energy balance through energy partitioning under low-temperature conditions.

Diverse responses to low temperature - herbaceous and woody plants do it differently

Plant acclimation to any change causing genotypic and phenotypic alterations represents the temporal integration of short-term and long-term fluctuations in an environmental signal such as a change in temperature. In nature, acclimation to low temperature is usually initiated by a short-term fluctuation in temperature which perturbs metabolic homeostasis and induces a stress response. Depending on the amplitude, frequency and duration of the stress, acclimation to the new low temperature regime is the result of some long-term adjustment reflecting the development of a new homeostatic state. Plant species have evolved diverse strategies to cope with the consequences of stress and acclimation to cold temperatures (Öquist and Hüner, 2003). Herbaceous winter annuals exploit the autumn months to establish site occupancy involving the enhancement of photosynthetic carbon metabolism and coordinated changes in respiratory metabolism. Evergreen trees downregulate photosynthesis (Savitch *et al.*, 2002). This includes the inactivation of PSII reaction centres (RCs) and the re-organization of the light-harvesting complexes (LHC) from pigment-protein complexes efficient in light harvesting and energy transfer to complexes primed for energy quenching during autumn and winter (Ensminger *et al.*, 2004; Ottander *et al.*, 1995; Savitch *et al.*, 2002).

Physiological changes associated with photosynthesis can be directly characterized by CO_2/O_2 gas exchange and/or by chlorophyll fluorescence measurements. Under laboratory conditions, annual cold tolerant plants such as wheat, rye and *Arabidopsis thaliana* show a decrease in light-saturated rates of CO_2 uptake with minimal changes in apparent quantum yield for CO_2/O_2 uptake in response to a sudden shift from warm to cold temperatures. This is followed by a strong recovery of photosynthesis under cold temperatures, once leaves are acclimated (Öquist and Hüner, 1993; Hurry *et al.*, 2000). In contrast, the transfer of evergreen conifers such as *Pinus sylvestris* to cold temperatures caused a substantial decrease in photosynthesis but no recovery of photosynthesis under low temperature conditions, once plants were fully acclimated (Ensminger *et al.*, 2005; Savitch *et al.*, 2002; Sveshnikov *et al.*, 2006).

Data from field experiments reflect the much more complex growth environment experienced under natural conditions. In field grown *Brassica napus*, quantum yield of CO_2 uptake did not substantially change from October until November, when maximum temperatures decreased from about 20 °C to about 2 °C (Farage and Long, 1991). Only during mid-winter, when air temperature frequently dropped to below freezing and photosynthetic photon flux density received by the canopy caused photoinhibition, did the quantum yield of CO_2 uptake decrease substantially. Nonetheless, CO_2 uptake always recovered within days when air temperatures intermittently increased above 2 °C. This is consistent with controlled laboratory experiments and the fact that photoperiod has minimal affects on photosynthetic acclimation to low temperatures in herbaceous plants (Gray *et al.*, 1994; Hüner *et al.*, 1993).

Conifer photosynthesis also decreases suddenly upon exposure to low temperatures in the field. For example, field-grown *Picea abies* exhibited a decrease in maximum photochemical efficiency (F_v/F_m) during cold autumn conditions when air temperature was less than 5 °C (Lundmark *et al.*, 1998). However, during a subsequent much warmer autumn, there was no sudden decrease in Fv/Fm, and values remained close to summer levels until the end of October (Lundmark *et al.*, 1998). These data suggest that temperature alone has a major impact on the timing of seasonal changes in photochemical efficiency and that photoperiod has minimal affects on the downregulation of photosynthesis in field-grown conifers. Studying the effects of photoperiod and temperature

on the autumn transition in *Pinus banksiana* seedlings under controlled experimental conditions, we found low temperature to be the important trigger for the autumn downregulation of photochemical efficiency and the degradation of PSII RC protein D1 as compared with photoperiod (Fig. 6). This view is strongly supported by whole canopy gas-exchange data from different Scots pine forests. Under boreal winter conditions, net photosynthesis in Scots pine (*P. sylvestris*) is completely suppressed during subfreezing temperatures from October until about mid-May (Ensminger *et al.*, 2004; Lloyd *et al.*, 2002). In a maritime ecotype Scots pine forest in the Netherlands, whole canopy gas-exchange measurements also indicated the autumn downregulation of photosynthesis (Dolman *et al.*, 2002). However, in contrast to controlled laboratory studies, Dolman *et al.* (2002) also observed a substantial recovery of photosynthetic net carbon uptake of this Scots pine forest during warm winter days in January. Such field data for the recovery of photosynthesis from mature Scots pine forests seem to contradict the results obtained from young seedlings under controlled laboratory conditions (Öquist and Hüner, 2003; Savitch *et al.*, 2002; Sveshnikov *et al.*, 2006). However, the magnitude of recovery of photosynthesis under field conditions during wintertime under temperate climate conditions is much smaller than photosynthetic gain observed under summer conditions (Dolman *et al.*, 2002).

On the basis of field and laboratory observations, Bauer *et al.* (1994) proposed two distinct strategies in woody and herbaceous plants to cope with low temperatures. Long-lived woody plants give priority to a high degree of frost resistance including increased photoprotective systems over efficiency of photosynthesis to survive the winter. In contrast, they propose that short-lived herbaceous plants retain a higher photosynthetic activity and adjust photosynthesis to maximize carbon gain at the risk of frost damage. As discussed in detail by Öquist and Hüner (2003), it is also important to consider the fact that conifers retain their needles for some years, whereas the leaves of coldtolerant, overwintering crop plants such as wheat and rye, die in the subsequent spring. This gives rise to new leaves which are acclimated to spring growth conditions under both controlled environment and field conditions (Hüner *et al.*, 1993; Savitch *et al.*, 2000). The initial energy for this early spring growth comes largely from stored carbohydrates in the crown tissues of these cereals. Thus, the persistence of conifer

needles is essential to their winter survival, whereas in cold tolerant cereals, the persistence of the crown as a carbon storage organ and not the leaves is essential for winter survival.

Photosynthesis as a low temperature sensor - a question of energy imbalance

Photosynthesis aims to maximize the use of the available light to optimize the use of carbon and nitrogen resources and to minimize the damaging effects of excess absorbed energy (Falkowski and Chen, 2003; Hüner *et al.*, 1998, 2003; Paul and Foyer, 2001). This involves feedback regulation of photosynthesis to maintain an energy balance between light absorbed by the primary photochemical reactions in photosystem (PS) II and PS I, the transformation of this energy into NADPH and ATP and finally its utilization in metabolism and growth (Fig. 7). This balance of energy flow between the photophysical and photochemical processes that transform light and the metabolic sinks that consume the energy is called photostasis (Öquist and Hüner, 2003). Balancing the energy flow through the process of photosynthesis is a challenge, because the extremely rapid, temperature-independent photochemical reactions are integrated with relatively slow, temperature-dependent biochemical reactions. Because the biochemical reactions limit the rate of energy flow, the thermodynamic constraints imposed by low temperatures exacerbate a potential energy imbalance by differentially affecting energy consumption by metabolism (Fig. 7).

Adjustment to imbalances in the energy flow from source to sink via electron transport requires a mechanism to sense cellular energy status. This is achieved in phototrophic cells through a redox sensor within the photosynthetic electron transport chain, the PQ pool (Allen and Nilsson, 1997; Escoubas *et al.*, 1995; Maxwell *et al.*, 1995; Pfannschmidt, 2003). When light energy is converted to redox potential energy, the rate-limiting step is the conversion of 'closed' RCs to 'open' RCs through oxidation of Q_A^- by the PQ pool and intersystem electron transport (Fig. 7). The primary quinone, Q_A, bound to PSII RCs is reduced by primary charge separation and is oxidized through electron transfer to the secondary bound quinone, Q_B. The double reduction and protonation of Q_B causes it to dissociate from PSII and enters the free PQ pool as reduced

plastoquinone (PQH_2). Because reduced Q_B is in equilibrium with the PQ pool, the reduction state of Q_A reflects the redox state of the free PQ pool. Excitation pressure or excessive excitation energy is a relative measure of the reduction state of Q_A and reflects the redox state of the intersystem PQ pool (Hüner *et al.*, 1998). This can be measured in vivo or in vitro by pulse amplitude-modulated fluorescence as $1-q_P$. Thus, excitation pressure is a measure of the imbalance in energy flow, that is, a measure of the extent to which $I > E_k$, and thus, $\sigma_{PSII}\, I > \tau^{-1}$ (Fig. 8).

As a primary energy sensor, the PQ pool transduces the electron transport signal into biochemical signals that regulate transcription of genes involved in cold acclimation in both the chloroplast and the nucleus. To maintain an energy balance, regulation of photosynthetic genes reflects either the adjustment of the source and hence primary photosynthetic reactions and redox components of the photosynthetic electron transport chain or the adjustment of the sink capacity and hence enzymes involved in chloroplastic and cytosolic carbon metabolism. Based on the inequality, $\sigma_{PSII}\, E_k > \tau^{-1}$, energy balance or photostasis ($\sigma_{PSII}\, E_k = \tau^{-1}$) upon a shift to low temperatures can be attained in the following ways: (1) by adjusting σ_{PSII} through a reduction in the functional absorption cross-section of PSII; (2) by adjusting σ_{PSII} through a decrease of the physical size or abundance of the major light-harvesting antenna complex associated with PSII RCs; (3) by decreasing the incident irradiance (I) such that $I > E_k$ and finally (4) by increasing the electron sink capacity (τ^{-1}) by upregulation of the rate of CO_2assimilation and the carbon metabolic pathway. It appears that photoautotrophs exploit any one or a combination of these mechanisms to balance the energy flow under low temperature conditions (Hüner *et al.*, 1998, 2003).

It has been presumed that it is a redox signal, which emanates from the PQ pool to regulate chloroplast and nuclear photosynthetic gene transcription (Allen and Nilsson, 1997; Escoubas *et al.*, 1995; Fey *et al.*, 2005a; Maxwell *et al.*, 1995; Pfannschmidt, 2003) as well as non-photosynthetic nuclear genes involved in cold acclimation (Hüner *et al.*, 1998). However, the thylakoid ΔpH can also act as an important chloroplastic signal emanating, in part, the PQ pool of the thylakoid membrane. The trans-thylakoid ΔpH is a key regulator of non-photochemical energy quenching through the xanthophyll cycle (Horton *et al.*, 1999; Krause and Weis, 1991).

Photoacclimation under low temperatures - mechanisms to balance energy flow

Exposure to either high light or low temperature can induce a comparable high excitation pressure which can result in photoinhibition, the light-dependent decrease in the photosynthetic rate. Rapidly reversible photoinhibition is a consequence of increased thermal dissipation of energy (or non-photochemical quenching, NPQ) leading to a decrease in the effective σ_{PSII} and downregulation of PSII activity (Öquist and Hüner, 1993). Primarily, this is a photoprotective mechanism that involves the xanthophyll cycle (Demmig-Adams and Adams, 1996). If the absorbed energy exceeds both the capacity of photochemistry as well as the capacity for NPQ, this results in photodamage because of the greater rate of destruction versus repair of the D1 PSII RC protein (Melis, 1999). Thus, photoacclimation to low temperature mimics photoacclimation to high light which attempts to balance the energy absorbed versus the energy either utilized through metabolism or dissipated via NPQ to avoid photodamage.

Functional absorption cross-section of PSII

Photoacclimation at the level of the energy source may involve adjustments in the efficiency of energy transfer from LHC to PSII RCs without changing the physical size of the LHC. An important, rapid mechanism to balance energy flow is through dissipation of excess energy via NPQ within the LHC. NPQ via the antenna occurs through the xanthophyll cycle, the reversible, light-dependent conversion of the light-harvesting pigment, violaxanthin, to the quenching pigments, antheraxanthin and zeaxanthin, which are unable to pass their excitation energy to Chl a (Horton *et al.*, 1999; Niyogi *et al.*, 2005). Thus, the induction of the xanthophyll cycle modulates excitation pressure by affecting the effective absorption cross-section of PSII (σ_{PSII}) to protect PSII from over-excitation and photodamage. Holt *et al.* (2005) have provided evidence that the direct quenching of excess excitation energy via NPQ occurs through the formation of a carotenoid radical cation upon excitation of chlorophyll under conditions of maximum, steady-state NPQ. Thus, the xanthophyll cycle acts as a 'molecular gear shift' regulating energy transfer within the LHC (Frank *et al.*, 1994). In addition to this dynamic modulation of excitation pressure, many overwintering plants develop sustained,

xanthophyll-dependent energy dissipation (Demmig-Adams and Adams, 1996). This is an important protective mechanism enabling evergreen plants to maintain their leaves during the winter. Many mesophytic overwintering plants such as *Malva*, *Arabidopsis* and winter cereals exhibit a 'cold-sustained' NPQ which is rapidly reversible upon warming. In contrast to this reversible form, schlerophytic evergreens also exhibit a sustained form of zeaxanthin-dependent NPQ which predominates during the winter and is not rapidly reversible upon warming. This persistent quenching appears to be associated with the re-organization of the LHC of PSII (LHCII) into xanthophyll-containing aggregates induced by a combination of low temperature and high light (Gilmore and Ball, 2000; Öquist and Hüner, 2003). However, this persistent type of sustained quenching has not been reported for mesophytic plant species (Adams *et al.*, 2002).

An alternative mechanism to affect functional σ_{PSII} by modulating energy transfer is to decrease the connectivity between the peripheral light-harvesting system and the internal core antenna complexes that transfer the energy directly to PSII RCs. This is observed in green algae (Wilson *et al.*, 2003) which exhibit detached LHCs when grown under high excitation pressure. This decrease in energy-transfer efficiency results in an increase in the fluorescence yield of Chl a at 680 nm characteristic of free LHCII (Krause and Weis, 1991). Thus, the increase in absorbed energy is re-emitted as light and consequently less energy transferred to PSII RCs for charge separation.

Antenna size and organization

Within the time scale of minutes, photosynthesis can adjust to compensate for high excitation pressure created by sudden changes in light and/or temperature by reducing energy-transfer efficiency to PSII through diverting energy from PSII to PSI via state transitions. When PSII is preferentially excited relative to PSI, the redox sensor PQ becomes reduced, which activates a thylakoid protein kinase that phosphorylates LHCII. As a consequence of charge repulsion, a population of peripheral LHCII migrates and associates with PSI via the PSI-H subunit (Lunde *et al.*, 2000). This re-distributes the light-harvesting chlorophyll to PSI at the expense of PSII and results in a balanced excitation of PSII and PSI to ensure optimal quantum efficiency for photosynthetic electron

transport. Recently, Depège *et al.* (2003) isolated a chloroplast protein kinase, Stt7, in the green alga *Chlamydomonas reinhardtii*. This short-term response to sudden changes in excitation pressure is reversed when PSI is preferentially excited relative to PSII. Under these conditions, the PQ pool is oxidized, the LHCII kinase is inactive, and phosphorylated LHCII is deposphorylated by a thylakoid protein phosphatase. As a consequence, LHCII re-associates with PSII to re-distribute energy for a balanced excitation of both photosystems (Allen and Nilsson, 1997; Haldrup *et al.*, 2001). Thus, in contrast to photoprotection via either xanthophyll cycle or LHCII detachment, state transitions affect σ_{PSII} by modulation of the physical size of LHCII associated with PSII. On a longer time scale, σ_{PSII} can be modulated by affecting the physical size of LHCII per PSII RC. This occurs through the regulation of transcription and translation of the Lhcb family of nuclear genes encoding LHCII polypeptides. Thereby, PSII is protected from excessive excitation by decreasing the probability of light absorption through the development of a smaller LHC. This is reflected in increases in the Chl a/b ratio and a decrease in the quantum yields for CO_2 fixation and O_2 evolution. Such photoacclimation to light or low temperatures was observed in conifers and certain herbaceous plants (Sveshnikov *et al.*, 2006; Walters and Horton, 1994) as well as in green algae (Escoubas *et al.*, 1995; Maxwell *et al.*, 1995). In cold acclimated winter pine, PSI photochemistry is less inhibited than PSII photochemistry, enhancing cyclic electron transfer around PSI that can provide electrons to synthesize ATP under conditions where the rates of photosynthetic linear electron transport are limited at the level of PSII (Ivanov *et al.*, 2001).

In contrast to the 'molecular gear shift hypothesis' for the zeaxanthin-dependent NPQ, it has been proposed that the *trans*-thylakoid ΔpH, and the xanthophyll cycle pigments regulate the oligomeric state of LHCII which affects the rapidly relaxing energy-dependent component (q_E) of NPQ (Horton *et al.*, 1999). In support of this mechanism, Elrad *et al.* (2002) reported that LHCII trimerization is required for antenna quenching in the npq5 mutant of *C. reinhardtii*. The seminal work of Trémolières and coworkers showed that phosphatidylglycerol (PG), the major phospholipid present in chloroplast thylakoid membranes, and its fatty acid composition play a crucial role in stabilizing the oligomeric or native trimeric state of LHCII (Dubacq and Trémolières, 1983). Hüner

and coworkers showed that acclimation of rye to low temperature resulted in a specific decrease in the content of PG containing *trans*-Δ^3-hexadecenoic acid (*trans*16:1) with a concomitant shift in the supra-molecular organization of LHCII from its oligomeric to monomeric state (Hüner *et al.*, 1987, 1989). Chloroplast biogenesis showed that LHCII is inserted into the thylakoid membrane in its monomeric form which subsequently is stabilized in its oligomeric form (Krol *et al.*, 1988, 1989). Are the levels of *trans*16:1 and the aggregation state of LHCII regulated by excitation pressure? Increasing the growth irradiance of rye (*Secale cereale* cv Musketeer) from 50 to 800 μmol photons $m^{-2} s^{-1}$ at 20 °C resulted in a 1.8-fold increase in the *trans*16:1 content with a concomitant decrease in hexadecanoic acid levels in PG which favoured a preponderance of aggregated LHCII measured in vitro as the ratio of oligomeric:monomeric LHCII (Gray *et al.*, 2005). During growth at 5 °C, similar irradiance-dependent increases in the *trans*16:1 content and the aggregation state of LHCII were observed. However, low temperature growth resulted in 1.4-fold lower transhexadecenoic acid content and a lower LHCII oligomer : monomer ratio compared to growth at 20 °C and at the same irradiance. These trends were also observed under natural field conditions for 10 cultivars of rye, six cultivars of barley and four cultivars of wheat. Thus, the accumulation of *trans*16:1 as well as the aggregation state of LHCII are modulated by both growth irradiance and growth temperature in an independent but additive manner and not by excitation pressure under both controlled environment and field conditions.

RC quenching

Although the major focus of recent research on photoprotection has been on feedback de-excitation through antenna quenching involving PsbS and the xanthophyll cycle (Niyogi *et al.*, 2005; Holt *et al.*, 2005) and state transitions (Lunde *et al.*, 2000; Depège *et al.*, 2003), quenching of excess energy can occur independently of zeaxanthin (Ivanov *et al.*, 2001, 2002; Lee *et al.*, 2001; Sane *et al.*, 2003), supporting a role for RC quenching in photoprotection of PSII as originally proposed by Briantais *et al.* (1979) and Krause and Weis (1991). Ivanov *et al.* (2001) reported that the activation energies for PSII charge recombination decrease under high excitation pressure and occur independently of antenna quenching as well as state transitions. Thus, PSII RC quenching represents an

important component of NPQ in all photoautotrophs and complements antenna quenching via PsbS and the xanthophyll cycle. However, in contrast to the rapidly reversible antenna quenching (Horton *et al.*, 1999), the capacity for PSII RC quenching is retained during steady-state growth at high excitation pressure and develops more slowly on a time scale of days to weeks in *A. thaliana* (Sane *et al.*, 2003) and pine (Ivanov *et al.*, 2002; Sveshnikov *et al.*, 2006). In the field PSII, RC quenching in pine is maximum at low temperatures during the winter months when pine needles are photoinhibited but decreases to minimum upon spring recovery in late spring when pine needles exhibit maximum photosynthetic activity (Ivanov *et al.*, 2002).

Stoichiometry of the photosynthetic apparatus

The modulation of photosystem stoichiometry is a response to changes in the redox state of the intersystem electron transport chain to ensure equal rates of electron flow through both PSI & PSII. Pfannschmidt *et al.* (1999) have shown that the transcription of the chloroplast encoded *psbA* which codes for D1 and *psaAB* genes which code for the PSI RC polypeptides is controlled by the redox state of the PQ pool. Over-reduction of the PQ pool by the preferential excitation of PSII favours not only the energy transfer from PSII to PSI through phosphorylation of LHCII but also the activation of *psaAB* transcription and the concomitant repression of *psbA*. Conversely, oxidation of the PQ pool by preferential excitation of PSI favours not only the de-phosphorylation of LHCII but also the activation of transcription of *psbA* and the repression of *psaAB* (Pfannschmidt *et al.*, 1999). Thus, PQ, the redox sensor that controls state transitions, also appears to be the sensor that regulates chloroplast photosystem stoichiometry.

Cold-induced aggregation of components of the photosynthetic apparatus

Cold acclimation of conifers induces cessation of primary growth, thereby decreasing the sink demand for photoassimilates which decreases the capacity to utilize energy metabolically (τ^{-1}). This results in feedback inhibition of photosynthesis (Hjelm and Ögren, 2003; Savitch *et al.*, 2002). To attain photostasis under these conditions, conifers exhibit long-term changes in the organization of the photosynthetic apparatus (Ebbert *et al.*, 2005; Ensminger *et al.*, 2004; Ottander *et al.*, 1995; Savitch *et al.*, 2002). Ever-

green conifers exhibit an unusual response to cold acclimation and over-wintering conditions with a decrease in number of PSII RCs, a loss of light-harvesting chlorophylls and a unique, aggregated state involving LHCII, PSII and PSI. This aggregation state seems to be associated with increased levels of PsbS and the formation of a sustained NPQ via increased levels of zeaxanthin. The formation of this winter-induced, aggregated state is fully reversible in the spring (Ottander *et al.*, 1995) with the resumption of photosynthesis without the immediate de novo synthesis of chlorophyll (Öquist and Hüner, 2003). Under natural field conditions, initially high PsbS levels can decrease upon exposure to severe subfreezing temperatures together with concomitant loss of functional PSII, once trees have developed the fully acclimated, photoinhibited state (Ebbert *et al.*, 2005; Ensminger *et al.*, 2004). Thus, the re-organization of the photosynthetic apparatus of pine leads to a highly quenched state that protects conifer needles from excess absorbed energy by dissipating it as heat (Öquist and Hüner, 2003). As a consequence, conifer needles remain green in the winter. However, it is not established if the redox state of the PQ pool plays any role in this reorganization of conifer thylakoids.

A functional analysis of the photosynthetic apparatus of snowgum (and the hemiparasitic mistletoe (*Amyema miquelii* Lehm. ex Miq.) indicates that these evergreens undergo a similar re-organization of the photosynthetic apparatus. Under winter field conditions, these evergreens exhibited a feature of the Chl a fluorescence spectra called the cold-hard-band (Gilmore and Ball, 2000), which contributes to photoprotection of PSII by dissipating absorbed energy as heat. In contrast to evergreens, winter cereals such as rye continue to grow and develop during the cold acclimation period to attain maximum freezing tolerance, thereby maintaining a high demand for photoassimilates during the cold acclimation process. These plants maintain maximum efficiency and capacity for light absorption (σ_{PSII}) as indicated by increased Chl per leaf area, minimal changes in Chl a/b ratios in the relative contents of Lhcb1, Lhcb2, D1, Cytf, PC, PsaA/PsaB heterodimer and the b-subunit of the ATPase complex on a Chl basis (Gray *et al.*, 1998). Winter rye exhibits a twofold increase in thylakoid plastoquinone A content which is associated with increasing excitation pressure and a concomitant increase in the apparent size of the intersystem electron donor pool to PS I. Because excitation

pressure regulates this response in rye, it is presumed that the redox state of the PQ pool is the major sensor controlling the size of the PQ pool (Gray *et al.*, 1998).

Acclimation of electron sink capacity

While conifers reduce their needs for assimilates when entering the dormant period with the cessation of growth and thus decreased sink capacity (τ^{-1}), herbaceous plants such as winter wheat continue to grow and maintain their need for photoassimilates even after cold acclimation (Savitch *et al.*, 2002; Leonardos *et al.*, 2003). Such different strategies of cold acclimation in conifers and cold hardy herbaceous plants underscore the importance of both source and sink as control points to regulate photosynthesis under low temperatures to maintain photostasis (Hüner *et al.*, 1998; Paul and Foyer, 2001).

Primary carbon metabolism, carbon allocation and partitioning

Optimal photosynthesis requires a balance between the rates of carbon fixation in the chloroplast and cytosolic sucrose synthesis (Fig. 9). Excessive sucrose synthesis depletes the phosphorylated Calvin cycle intermediates and inhibits regeneration of RuBP. Conversely, inadequate sucrose synthesis leads to accumulation of phosphorylated intermediates and depletion of Pi, resulting in inhibition of ATP synthesis, accumulation of glycerate-3-P and inactivation of Rubisco. Thus, photosynthesis requires strict regulation of carbon allocation between chloroplastic and cytosolic carbon metabolism.

Cold hardy herbaceous plants show different responses when exposed to cold temperature. It is important to distinguish whether leaves first fully developed under warm conditions and then were shifted to colder conditions or whether leaves were developed under cold conditions only (Gray and Heath, 2005). Low-temperature stress inhibits sucrose synthesis in the cytosol causing decreased Pi cycling between the cytosol and the chloroplast (Hurry *et al.*, 2000). Thus, the chloroplast becomes Pi-limited impeding the synthesis of ATP which is needed in the regeneration of RuBP. This decreases the rates of electron transport and causes feedback inhibition of photosynthesis (Hurry *et al.*,

2000). Under these conditions, excitation pressure increases because of limitations in sink capacity (τ^{-1}).

In contrast, growth and development at low temperatures increase the expression and subsequent activity of Calvin cycle enzymes (Hurry *et al.*, 2000)). In addition, cold-tolerant, herbaceous plants grown at cold temperatures exhibit an increase in Pi availability in the chloroplast (Stitt and Hurry, 2002), as well as in adenylates and phosphorylated intermediates, and in the capacity for the regeneration of RuBP (Hurry *et al.*, 1994). Cold grown plants also exhibit an increase in sucrose-phosphate- synthase (SPS) activity, SPS activation state as well as an increase in the cytosolic hexose-P pool leading to sucrose biosynthesis (Stitt and Hurry, 2002).

Concomitant with the upregulation of carbon metabolism, cold acclimated winter wheat also exhibits a stimulation of carbon export from the leaf compared to cold-stressed plants (Leonardos *et al.*, 2003). This is reflected in an increased capacity to assimilate CO_2 and an increase in plant biomass with minimal changes in photosynthetic efficiency measured as the apparent quantum yield of either CO_2 fixation or O_2 evolution (Gray *et al.*, 1997; Öquist and Hüner, 1993; Savitch *et al.*, 2002). In addition, this increased biomass production and carbon export capacity occur with significant changes in plant morphology, leaf anatomy and stomatal distribution (Strand *et al.*, 1999).

Upregulation of carbon metabolism under low temperatures is linked to decreased inhibition of photosynthesis

The above-mentioned alterations also result in increased water use efficiency in cold acclimated winter wheat plants (Leonardos *et al.*, 2003) which is interesting, because cold acclimation has been shown to enhance resistance to dehydration as well as freezing stress (Thomashow, 1999). The extent to which cereals are able to increase their photosynthetic capacity during cold acclimation is directly correlated to their freezing tolerance measured as LT50 (Öquist and Hüner, 1993). Thus, cold-tolerant winter cereals maintain photostasis by upregulating sink capacity (τ^{-1}) and carbon export while keeping σ_{PSII} relatively constant compared to cold-stressed plants (Leonardos *et al.*, 2003).

In an elegant series of experiments, Strand *et al.* (2003) tested the role of the upregulation of sucrose biosynthesis on freezing tolerance by comparing cold acclimation in wild-type *A. thaliana* with transgenic plants over-expressing SPS or with antisense repression of either cytosolic fructose-1,6-bisphosphatase (cFBPase) or SPS. Over- expression of SPS improved photosynthesis, increased the flux of fixed carbon into sucrose and increased freezing tolerance during a cold stress relative to wild-type and the antilines examined. Interestingly, the recovery of photosynthesis typically observed for *Arabidopsis* leaves developed at low temperature was inhibited in the antisense lines which also failed to cold acclimate. Strand *et al.* (2003) conclude that an increased capacity for sucrose biosynthesis at low temperature reduces the inhibition of photosynthesis when coupled to the mobilization of carbohydrates from source leaves to sinks and increases the rate at which freezing tolerance develops in *Arabidopsis*.

Concomitant with the upregulation of carbon metabolism is the suppression of photorespiration in cold acclimated winter wheat (Savitch *et al.*, 2000). However, the suppression of photorespiration at low temperature was not exhibited in plants grown at high light and comparable excitation pressure. Thus, the suppression of photorespiration exhibited in cold acclimated winter wheat does not appear to be a response to excitation pressure but rather a specific response to low temperature (Savitch *et al.*, 2000).

Studies of non-hardy plants such as *B. napus*, tobacco and cold girdled spinach suggest that sink strength regulates photosynthetic capacity and that sugars act as a signal to repress photosynthetic gene expression (Stitt and Hurry, 2002). Strand *et al.* (1997) showed that long-term growth of *A. thaliana* at low temperatures increases soluble sugars in leaves without a concomitant inhibition of photosynthesis or repression of photosynthetic genes. They conclude that cold temperatures act as a signal in developing leaves to release the suppression of photosynthesis and gene expression. Hence, this allows photosynthesis to recover at low temperatures through increased activity and higher levels of photosynthetic and carbon metabolism enzymes. This recovery is accompanied by larger pools of phosphorylated intermediates which support high rates of metabolic carbon flux at low temperatures required for cold acclimation and the acquisition of freezing tolerance (Strand *et al.*, 2003). The release from the expected suppres-

sion of photosynthesis in overwintering herbaceous plants because of the accumulation of high levels of sucrose also reflects altered partitioning of carbon within the source leaf as well as to alternative sinks. For example, cold-acclimated wheat exhibits increased leaf vacuolar carbon storage through the polymerization of sucrose to fructans as well as increased fructan accumulation in the crown (Savitch *et al.*, 2000; Leonardos *et al.*, 2003). This is reflected in an increase in the volume of cytoplasm relative to vacuole, an increase in specific leaf weight and a decrease in leaf water content (Leonardos *et al.*, 2003; Stitt and Hurry, 2002).

There are very few studies that address the role of carbon flux and export during cold acclimation of woody species to low temperature. Savitch *et al.* (2002) reported that the inhibition of photosynthesis in pine during cold acclimation is associated with a cessation of carbon export from mature needles under controlled environment conditions. Hjelm and Ögren (2003) showed that although low temperatures create surpluses in carbohydrate in both trees and grasses, trees convert these surpluses to storage polysaccharides more efficiently than do grasses. Thus, grasses tend to accumulate more soluble carbohydrate than do trees. Grasses also exhibit minimal capacity to adjust the photosynthetic apparatus of leaves developed at warm temperatures and subsequently exposed to low temperatures. Over time at low temperature, leaves developed at warm temperatures senesce and are replaced by new, cold-acclimated leaves developed at low temperature. Hjelm and Ögren (2003) suggest that these different strategies to acclimate to low temperature provide contrasting benefits. Grasses increase their freezing tolerance from the accumulation of soluble carbohydrates. In contrast, trees benefit from the rapid conversion of carbohydrate surpluses into storage polysaccharides such as starch by allowing photosynthesis to be regulated independently of growth, as growth rates are modulated by temperature.

Temperature sensing also occurs independent from photosynthesis

Although the chloroplast thylakoid PQ pool appears to play an important role in sensing temperature through energy imbalances (Hüner *et al.*, 1998; Karpinski *et al.*, 1999), there are also mechanisms that cells use to detect changes in temperature indepen-

dently of photosynthesis and the PQ pool. These temperature sensors appear to be sensitive to low-temperature-induced changes in the physical properties of membranes. One such mechanism in plants is the activation of a two-component sensing/signalling pathway by changes in plasmamembrane viscosity (Murata and Los, 1997). In plants, two component systems are involved in responses to growth regulators such as ethylene and cytokinins as well as to osmotic responses (Urao *et al.*, 2000). Murata and Los (1997) suggest that low temperatures can be sensed in plants by two-component systems through temperature-induced changes in plasmamembrane fluidity. They propose that changes in membrane lipid microdomains cause a conformational change in a membrane-bound histidine kinase. This conformational change in the histidine kinase autophosphorylates a response regulator that, in turn, regulates the expression of desaturase genes involved in altering membrane fatty acid unsaturation. Although such a temperature-sensing two-component system has yet not been found in plants, a histidine kinase (Hik33) that is responsive to membrane viscosity has been detected in the outer membrane of the cyanobacterium, *Synechocystis* (Suzuki *et al.*, 2001; Murata and Los, 2006).

Monroy and Dhindsa (1995) have suggested an alternative low-temperature-sensing mechanism activated by changes in membrane viscosity. They suggest that plasmamembrane Ca^{2+} channels act as temperature sensors. According to this mechanism, a low-temperatureinduced decrease in membrane viscosity opens Ca^{2+} channels allowing the net influx of calcium into the cytosol. The increase in cytosolic Ca^{2+} triggers a signal transduction pathway that activates low temperature inducible nuclear genes. Microarray analyses indicate that not all cold-inducible genes are regulated by Hik33 in *Synechocystis* (Suzuki *et al.*, 2001) which is consistent with the thesis that several cellular mechanisms must exist to sense cold and that organisms most likely integrate a battery of low-temperature sensors to respond to fluctuating temperatures (Browse and Xin, 2001).

Interactions between photosynthetic redox and cold acclimation-signalling pathways

Although the signalling of redox regulation is still not clear, there is consensus that the redox state of the PQ pool regulates short- and long-term photosynthetic acclimation responses (Escoubas *et al.*, 1995; Falkowski and Chen, 2003; Hüner *et al.*, 1998; Maxwell *et al.*, 1995; Pfannschmidt, 2003). Recently, Carlberg *et al.* (2003) characterized a 9 kDA protein TSP9, which putatively is the signal from the thylakoid membrane that is transported to the RNA polymerase. This small redox-mediating signalling factor is associated with PSII and is partially released from the complex under reducing conditions. In addition to the role of TSP9 in retrograde redox signalling, there is evidence for several redox signals derived from the electron-transport chain via, e.g. reactive oxygen species, the redox state of PQ (Wilson *et al.*, 2003; Fey *et al.*, 2005a). Wilson *et al.* (2003) also demonstrated that the accumulation of Mg-protoporphyrin IX may act as an additional signalling component originating from the chloroplast regulating the accumulation of LHC polypeptides at low temperatures. Macroarray analysis actually identified 286 genes responding to the photosynthetic redox signal in *Arabidopsis* Fey *et al.* (2005b). The affected genes were not restricted to photosynthesis but included gene classes coding for gene expression, metabolism and signal transduction.

There is evidence for an interaction between the *COR* gene expression originating from a cold-induced signalling pathway and the redox state of the chloroplast. *COR*14 mRNA accumulates in barley grown under cold in the dark, but the COR14 protein accumulates in the chloroplast stroma only after brief exposure to light (Crosatti *et al.*, 1995). A recent study showed that the redox state of PQ actually promotes the accumulation of COR14B protein in the light, while the steady-state level of *COR*14 mRNA is independent from the redox state (Dal Bosco *et al.*, 2003). This reflects post-transcriptional modification of the transcript of the coldinduced *COR* gene which in turn is triggered through the DREB/CBF transcription factor.

A characteristic of cold acclimation in herbaceous plants is the accumulation of soluble sugars. Thus, sugar-signalling pathways are likely to be integral to both photosynthetic acclimation as well as cold acclimation. In *Arabidopsis*, the expression of nuclear-encoded photosynthetic genes (e.g. CAB2 and rbcS) is inversely correlated with intercel-

lular soluble sugar levels (Oswald *et al.*, 2001). This pattern is specific for photosynthesis genes, while nitrate reductase is positively correlated with sugar levels. The inhibition of photosynthetic electron transport by 3-(3,4-dichlorophenyl)-1,1-dimethylurea in sucrose-starved cells abolishes starvation-induced increase in CAB and rbcS transcript but not NR transcript abundance. This suggests starvation-induced increase in transcript levels of nuclear-encoded genes requires photosynthetic electron transport and is modulated through a plastid-derived signal (Oswald *et al.*, 2001)). Thus, plastid redox state regulates nuclear gene expression and actually overrides the sugar signal. When soluble sugar levels rise, e.g. because of an impairment of phloem loading by either genetic or environmentally determined growth cessation as under low temperatures, synthesis of new photosynthetic apparatus will be curtailed until the demand of carbon increases. Therefore, Oswald *et al.* (2001) suggest an interaction between sugar, photosynthetic electron transport and abscisic acid (ABA)-derived regulatory mechanisms to optimize photosynthetic gene expression in response to demand.

Photostasis and cold acclimation reflect a continuum of time-nested processes - conclusions and future perspectives

Regulation of photosynthesis in response to low temperatures maintains photostasis, the balance between energy absorbed by the source and energy used by metabolic sinks. Depending on the magnitude and duration of environmental change, photosynthesis exerts different mechanisms to overcome any energetic imbalance between source and sink. As suggested by Falkowski and Chen (2003), the transduction of the excitation pressure signal is mediated by a nested set of responses as a result of cellular integration over various time scales. On the biologically short-time scale of seconds to minutes, the reduction state of the PQ pool or some other component of the electron-transport chain may increase because of a sudden increase in irradiance or a sudden decrease in temperature or nutrient availability. The nested signal hypothesis (Falkowski and Chen, 2003) predicts that such short-term, abiotic stresses will result in a rapid increase in NPQ through the induction of the xanthophyll cycle and/or state transitions which will reduce the effective absorptive cross-section of PSII and maintain photostasis. However, if excitation pressure continues to increase over longer time periods of

hours to days, these short-term photoprotective processes will not be able to keep the PQ pool sufficiently oxidized and, as a consequence, will be superseded by longer term adjustments involving the downregulation of *Lhcb* expression in eukaryotes and the induction of RC quenching.

Clearly, there is complex cross-talk between the photosynthetic redox, cold acclimation, and sugar-signalling pathways to regulate overall plant acclimation to low temperature. In addition, there are overlapping relationships between cold acclimation and responses to dehydration and salt stress in terms of the biochemical changes and in the transcriptional regulation of the responses (Browse and Xin, 2001; Kacperska, 2004). Although the perception of low temperatures and transduction of the signals that initiate the suite of responses are still incomplete, there is evidence for multiple signalling pathways and probably multiple temperature sensors being used in higher plants to initiate and control cold acclimation. Thus, the process of cold acclimation includes the increased expression of many genes, decrease or cessation of growth, increases in ABA concentrations and changes in lipid membrane composition, accumulation of osmolytes including soluble sugars and increased levels of antioxidants (Browse and Xin, 2001; Kacperska, 2004). Just as photosynthetic acclimation to changes in light and temperature reflects a time nested response so too must the process of cold acclimation which integrates a complex suite of sensors and interacting signalling pathways (Stitt and Hurry, 2002).

Microarray transcript analyses provides the potential to assess differential gene expression for nutrient stress (Scheible *et al.*, 2004), desiccation stress (Collett *et al.*, 2004), drought and cold stress (Shinozaki *et al.*, 2003), photosynthetic redox regulation (Singh *et al.*, 2004) as well as the interaction of photosynthesis and cold acclimation (Savitch *et al.*, 2005). The power of this type of analysis lies in the simultaneous assessment of thousands of genes using microchip technology and statistical data analysis tools. However, there are still potential difficulties in the interpretation of the massive data sets focused on plant acclimation and stress-induced transcript analyses. As discussed above, acclimation and stress involve the integration of a complex suite of sensing and signalling pathways that respond in a time-dependent manner. Thus, for proper interpretation of such results, it is imperative that the experimental

design used to generate such complex data sets includes proper controls that allow one to segregate the interactive effects of various stresses. For example, because plants are photoautrophic, acclimation of plants to any given stress either under controlled environment or natural field conditions will involve light intensity effects that are independent of phytochrome. Because photostasis reflects cellular energy balance (σ_{PSII} $E_k = \tau^{-1}$), any abiotic or biotic stress will result in an imbalance in energy budget (I > E_k) usually because of limitations in τ^{-1}, the capacity to utilize the energy absorbed and transformed by photosynthesis. This imbalance will be reflected in high excitation pressure where σ_{PSII} $E_k > \tau^{-1}$. Thus, studies purporting to examine differential gene expression induced by changes in abiotic conditions such as irradiance, cold, nutrients or water availability need to account for the fact that a common component of all of these stresses is the potential to alter excitation pressure through modulation of the redox state of the PQ pool. For example, Gray *et al.* (1997) showed that cold acclimation and the development of freezing tolerance is a complex interaction of light, temperature and excitation pressure. In attempting to separate the effects of low temperature, irradiance and excitation pressure on gene expression in rye and wheat, Ndong *et al.* (2001) used a novel experimental design for macroarray analysis of 42 genes. Their design included cross-referencing five different controls within the same experiment aiming to minimize effects because of developmental differences. With this experimental design, the gene expression profiles of 37 of the 42 genes could be separated clearly into four distinct categories, which included gene regulation by temperature, irradiance, excitation pressure and the independent but additive effects of light plus temperature. Even this more complex experimental design exhibited limitations, because five of the 42 genes examined did not fit any of these categories (Ndong *et al.*, 2001). Although complex microarray experiments include considerable costs and generate huge data sets, we suggest that a similar approach must also be applied to large-scale gene expression analyses. With such an approach, we will begin to parse the complex interactive effects of excitation pressure, irradiance and stress and minimize the potential for misinterpretation of gene expression profiles.

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Figures

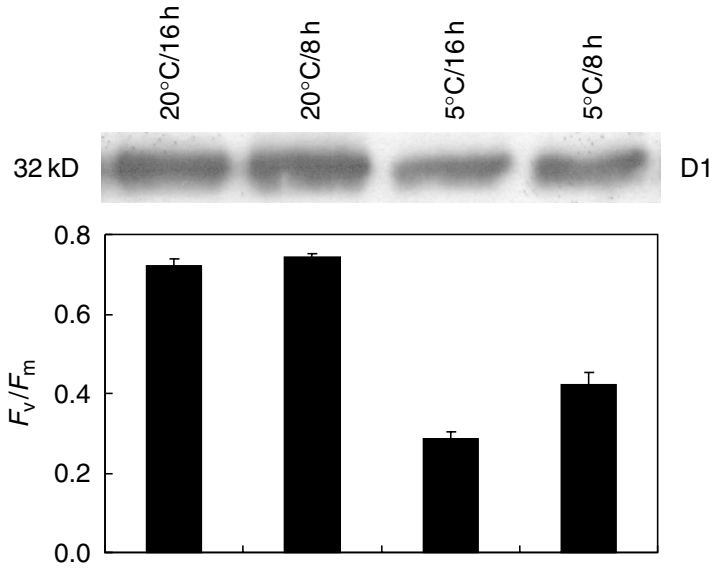

Figure 6: *Changes in photosystem II reaction centre protein D1 and the optimum quantum yield of chlorophyll fluorescence (F_v/F_m) in seedlings of* Pinus banksiana *4 weeks after transfer from summer (20°C/16 h photoperiod) to the indicated growth conditions. Decreases in D1 protein and F_v/F_m are because of growth at low temperature, whereas photoperiod does not have any effects*

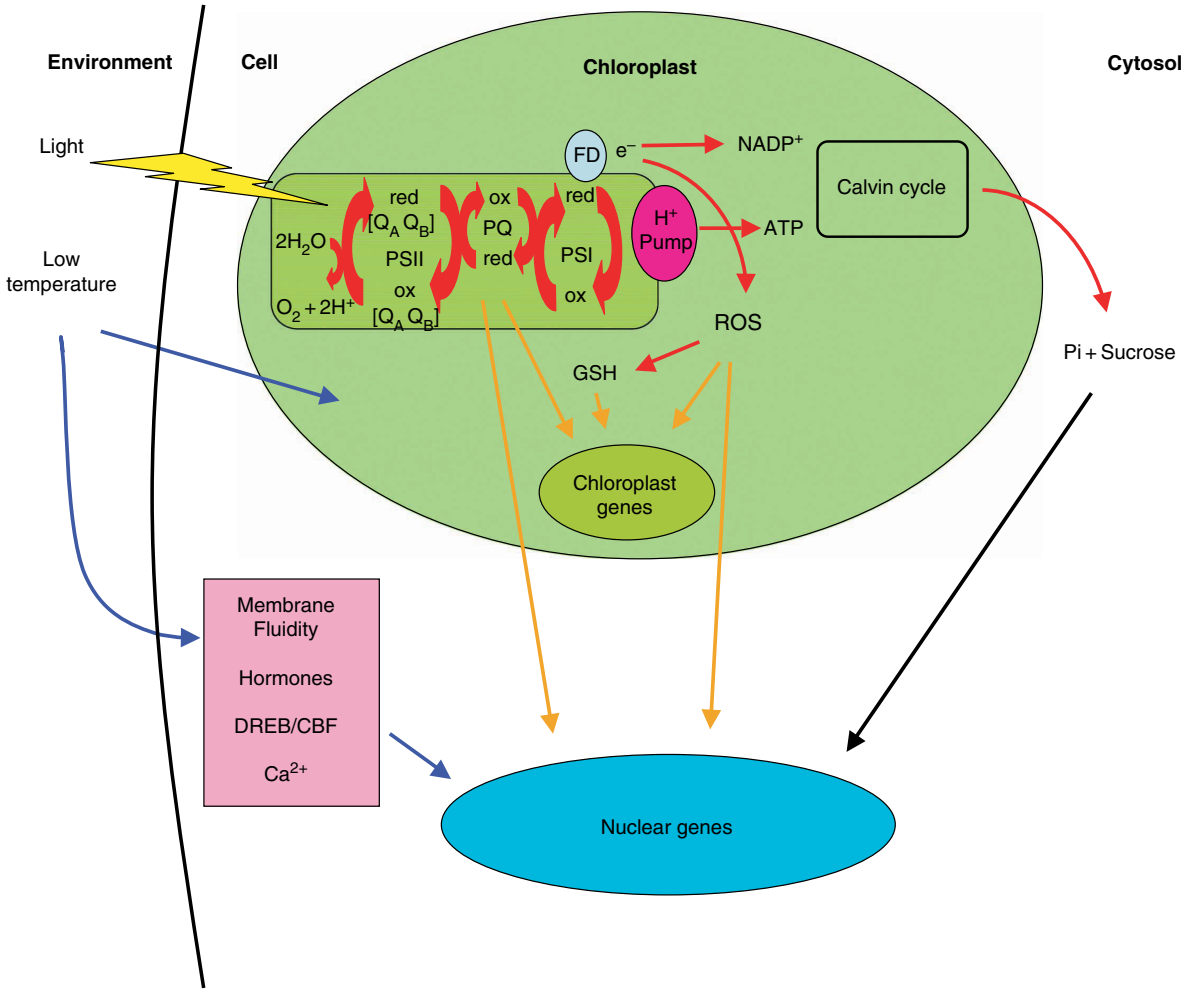

Figure 7: *Integration and regulation of the photosynthetic process. The primary light reactions occur within the thylakoid membrane system, where the water splitting complex, photosytem II (PSII) and PSI as well as the electron transport chain are located. Energy trapped by PSII is used to transfer electrons derived from the water-splitting process via the electron-transport chain by the mobile electron carrier plastoquinone (PQ) through linear electron flow. After a second light reaction in PSI the electrons can be accepted by $NADP^+$ via ferredoxin (FD). Protons are pumped across the thylakoid membrane to generate ATP. The main metabolic sink for NADPH and ATP is the Calvin cycle. Its sink strength depends on the rate of export of triose-P to the cytosol and subsequent export to sink tissues. Under increased excitation pressure, e.g. because of low temperature and/or high light intensities, electrons from PSI can also be transported to oxygen, thereby generating reactive oxygen species (ROS). ROS can be detoxified via gluthathione, causing changes in the ratio of the oxidized to the reduced state of gluthathione (GSH). Red arrows indicate the flow of energy, blue arrows indicate low temperature induced signals, orange arrows indicate redox-mediated signals and black arrows indicate sugar signals.*

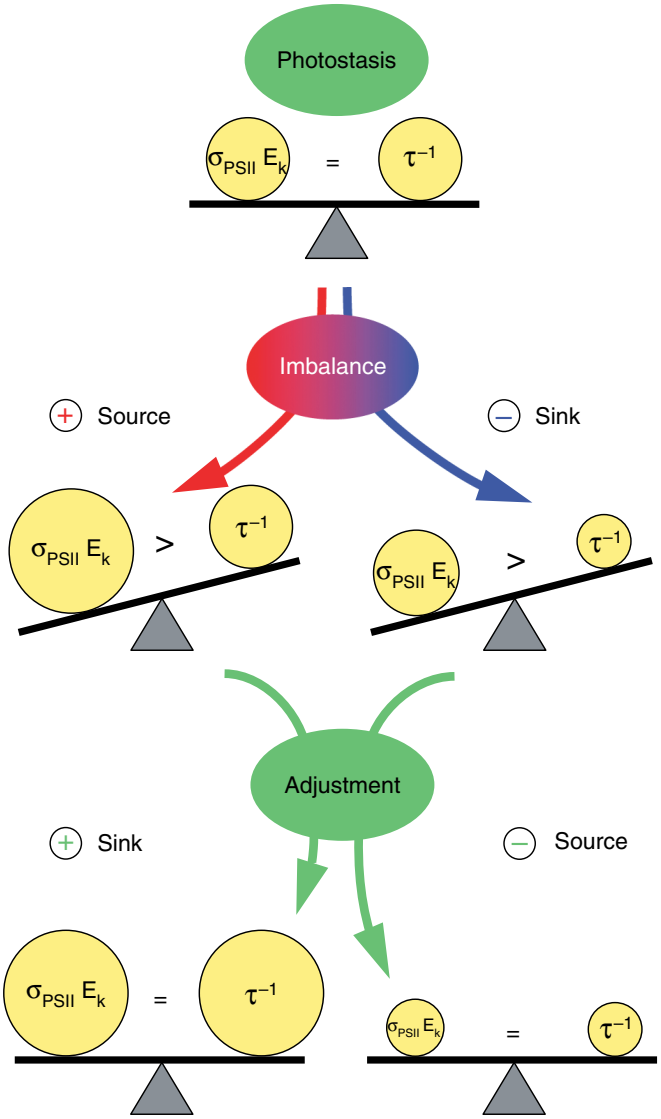

Figure 8: *Schematic illustration of the concept of photostasis as defined by the equation* $\sigma_{\mathrm{PSII}}\ \mathrm{E_k} = \tau^{-1}$ *(Falkowski and Chen, 2003). Photostasis is achieved through the balance of energy flow from sources to sinks where* σ_{PSII} *is the effective absorption cross-section of PSII,* $\mathrm{E_k}$ *is the irradiance (I) at which the maximum photosynthetic quantum yield balances photosynthetic capacity (estimated from a photosynthetic light response curve) and* τ^{-1} *is the rate at which photosynthetic electrons are consumed by a terminal electron acceptor such as* $\mathrm{CO_2}$ *under light-saturated conditions. An imbalance between energy absorbed versus energy utilized will occur whenever the rate at which the energy absorbed through PSII and the rate at which electrons are injected into photosynthetic electron transport exceeds temperature-dependent metabolic electron sink capacity (whenever* $\sigma_{\mathrm{PSII}}\ \mathrm{E_k} > \tau^{-1}$*). Such an imbalance can be created by increasing the growth irradiance to exceed* $\mathrm{E_k}$ *at a given* σ_{PSII} *(red arrow) or by lowering the growth temperature at a constant irradiance whereby* $\sigma_{\mathrm{PSII}}\ \mathrm{E_k} > \tau^{-1}$ *because of a temperature-dependent decrease in* τ^{-1} *(blue arrow). Adjustments of photosynthesis to balance the flow of energy and to obtain photostasis can either occur via an increase in the rate of sink processes, a decrease in the rate of energy provided through the source processes or a combination of both (green arrows).*

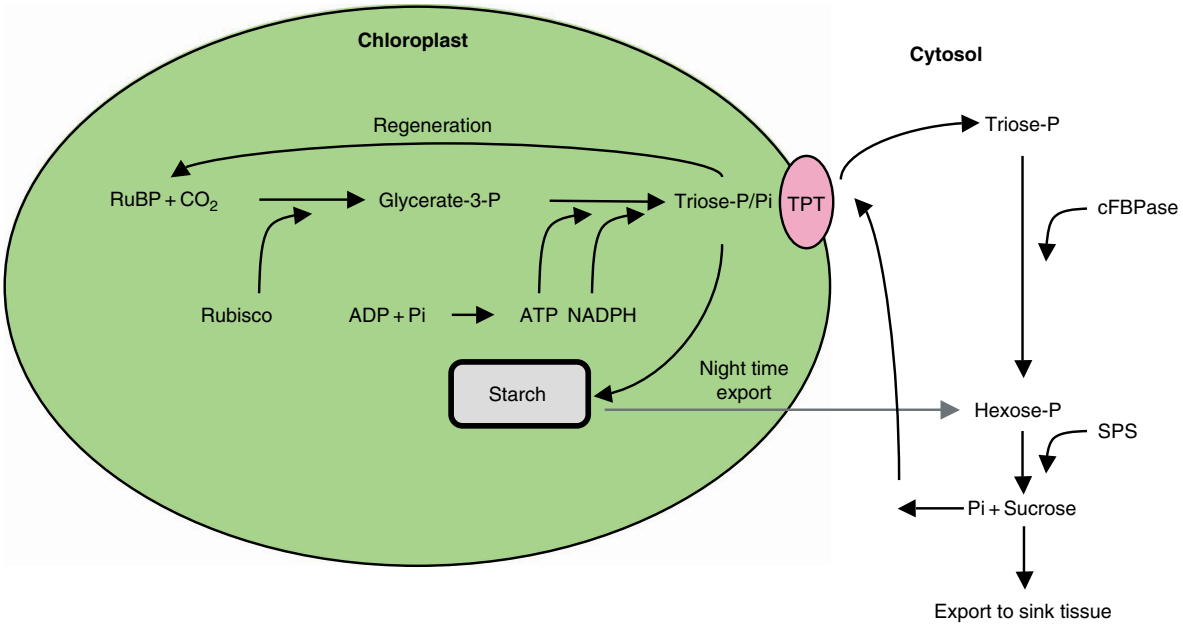

Figure 9: *Sugars act as metabolic sinks in the photosynthetic energy transformation process. Photosynthesis generates glycerate-3-phosphate (glycerate-3-P) which is reduced to produce triose-phosphate (triose-P) using NADPH and ATP. Most of the triose-P remains in the chloroplast to regenerate ribulose-1,5-biphosphate (RuBP). The surplus is converted to end products, thereby releasing inorganic phosphate (Pi) which is used to regenerate ATP. Export of triose-P via the triose-phosphate transporter (TPT) to the cytosol is the dominant end-product pathway. Triose-P is then converted to sucrose via cytosolic fructose-1,6-bisphosphatase (cFBPase) and sucrose phosphate synthase (SPS). When the rate of photosynthesis exceeds triose-P export, transient starch accumulates in the chloroplast and can be degraded during the night to maltose and glucose which are then exported to the cytosol (Zeeman et al., 2004). Cytosolic sucrose levels are under the control of (amongst other factors) the efficiency of assimilate export. Limited sink demand will cease sucrose export and thereby its biosynthesis resulting in decreased triose-P export from the chloroplast, accumulation of glycerate-3-phosphate and starch. The rate of starch biosynthesis can be an important safety valve to maintain high photosynthetic capacity when sink demand is limited because of, e.g. cold temperatures and low sugar export from source leaves, which favours the accumulation of soluble sugars in the cytosol.*

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Increased Air Temperature during Simulated Autumn Conditions Does Not Increase Photosynthetic Carbon Gain But Affects the Dissipation of Excess Energy in Seedlings of the Evergreen Conifer Jack Pine

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Abstract

Temperature and daylength act as environmental signals that determine the length of the growing season in boreal evergreen conifers. Climate change might affect the seasonal development of these trees, as they will experience naturally decreasing daylength during autumn, while at the same time warmer air temperature will maintain photosynthesis and respiration. We characterized the down-regulation of photosynthetic gas exchange and the mechanisms involved in the dissipation of energy in Jack pine (*Pinus banksiana*) in controlled environments during a simulated summer-autumn transition under natural conditions and conditions with altered air temperature and photoperiod. Using a factorial design, we dissected the effects of daylength and temperature. Control plants were grown at either warm summer conditions with 16 h photoperiod

and 22 °C or conditions representing a cool autumn with 8 h/7 °C. To assess the impact of photoperiod and temperature on photosynthesis and energy dissipation, plants were also grown under either cold summer (16 h photoperiod/7 °C) or warm autumn conditions (8 h photoperiod/22 °C). Photosynthetic gas exchange was affected by both daylength and temperature. Assimilation and respiration rates under warm autumn conditions were only about one-half of the summer values but were similar to values obtained for cold summer and natural autumn treatments. In contrast, photosynthetic efficiency was largely determined by temperature but not by daylength. Plants of different treatments followed different strategies for dissipating excess energy. Whereas in the warm summer treatment safe dissipation of excess energy was facilitated via zeaxanthin, in all other treatments dissipation of excess energy was facilitated predominantly via increased aggregation of the light-harvesting complex of photosystem II. These differences were accompanied by a lower deepoxidation state and larger amounts of β-carotene in the warm autumn treatment as well as by changes in the abundance of thylakoid membrane proteins compared to the summer condition. We conclude that photoperiod control of dormancy in Jack pine appears to negate any potential for an increased carbon gain associated with higher temperatures during the autumn season.

Introduction

Temperature and daylength are important drivers of physiological changes in boreal evergreen conifers because they determine the length of the growing season. In late summer and early autumn the decrease of the daylength is a signal that initiates cold hardening, a transition of physiological processes that allow hardy plants to survive severe winter conditions. The cold hardening process includes the cessation of growth and long-term changes in the metabolism of the tree (Weiser, 1970; Bigras *et al.*, 2001). Short days are supposed to induce the cold hardening most effectively, whereas decreasing temperatures affect this process only to some extent (Christersson, 1978).

As evergreen conifers keep their needles during winter, the cold hardening process involves a reorganization of the photosynthetic machinery (Ensminger *et al.*, 2004), as the needles retain a substantial amount of chlorophyll (Chl) and proteins and therefore continue to absorb light. Absorption of light under winter conditions can cause photoox-

idative damage of the photosynthetic apparatus, created by an imbalance between the photochemical generation of electrons and their diminished utilization due to decreasing sink capacity, i.e. the down-regulation of metabolism and growth in winter (Öquist and Hüner, 2003). To maintain the balance between light capture and energy utilization under conditions with altered sink capacity, energy flow and photosynthesis have to be adjusted by a process defined as photostasis (Öquist and Hüner, 2003). Plants have developed a wide range of mechanisms to achieve this balance and to avoid photooxidative damage. This includes adjustments of the Chl and protein concentration, changes in antenna size and organization, stoichiometry and aggregation of components of the photosynthetic apparatus, and a range of alternative energy dissipation pathways (Demmig-Adams *et al.*, 1996; Asada, 1999; Öquist and Hüner, 2003; Munekage *et al.*, 2004; Horton *et al.*, 2005; Kanervo *et al.*, 2005; Ensminger *et al.*, 2006). Recently, the carotenoid zeaxanthin in combination with the PSII subunit PsbS has gained interest as both play an important role in the safe thermal dissipation of excess energy via non-photochemical quenching (NPQ; Li *et al.*, 2000, 2002; Savitch *et al.*, 2002; Niyogi *et al.*, 2005). NPQ actually consists of three different components that are commonly defined by referring to their relaxation properties in darkness following a period of illumination (Müller *et al.*, 2001). A dynamic quenching mode is rapidly inducible and driven by the conversion of violaxanthin into zeaxanthin and protonation of the PsbS protein in response to acidification of the thylakoid lumen. State transition quenching relaxes within tens of minutes and is assumed not to be of great importance for photoprotection in terrestrial plants under high light (Niyogi, 1999; Kanervo *et al.*, 2005). The sustained quenching mode (q_I) in turn is induced in connection with a decrease in PsbS levels and a loss of functional PSII (Ensminger *et al.*, 2004; Ebbert *et al.*, 2005). In evergreen conifers, this q_I is also associated with the termination of growth and induction of frost hardiness (Öquist and Hüner, 2003). In this q_I state, a reorganization of the photosynthetic apparatus may protect conifer needles by dissipating excess energy as heat independent of a pH gradient (Gilmore and Ball, 2000; Horton *et al.*, 2005; Ensminger *et al.*, 2006). Overwintering evergreen conifers seem to be able to shift between the dynamic and the sustained antenna quenching (q_O) modes for dissipating excess energy (Öquist and Hüner, 2003). Aside from NPQ of excess energy in the antenna,

a zeaxanthin independent way of quenching has been described in the reaction center (RC; Ivanov *et al.*, 2001, 2006; Lee *et al.*, 2001; Sane *et al.*, 2003; Finazzi *et al.*, 2004). In addition to antenna and RC quenching, excess energy can be funneled into a range of alternative electron sinks, most notably photorespiration (Osmond and Grace, 1995; Streb *et al.*, 1998), the water-water cycle (Asada, 1999), and cyclic electron transport (Ivanov *et al.*, 2001; Kanervo *et al.*, 2005). For recent reviews, see Scheibe *et al.* (2005) and Ensminger *et al.* (2006).

Over the past decades substantial warming has occurred in northern latitudes, especially in the winter (Serreze *et al.*, 2000). It has been suggested that this is likely to increase the length of the growing season and thus the productivity of northern hemisphere forests (White *et al.*, 2000; Saxe *et al.*, 2001). Increased temperatures in autumn allow trees to maintain a higher quantum yield (Methy *et al.*, 1997). However, little data are available regarding the metabolic responses of evergreens of the boreal forests to extended growing seasons as a result of warmer air temperatures in late autumn. In this study, we focus on the interaction of altered autumn growth conditions and photosynthesis in Jack pine (*Pinus banksiana* Lamb.), an important evergreen conifer forming dominant stands on dry sandy soils in the western boreal zone of Canada, e.g. Northern Saskatchewan. Climate change will alter the phasing of temperature and daylength, the signals that trigger low temperature acclimation and development during autumn in conifer trees. The question is how the seasonal development is affected once trees experience the naturally decreasing daylength regulating growth cessation and limiting sink capacity while at the same time increased air temperature allows to maintain photosynthetic and respiratory capacity. Here, we used a factorial experiment with 16 h versus 8 h photoperiod (representing long day during July/August and short day during October/November, respectively, at e.g. 54 °N, 105 °W in northern Saskatchewan) and 22 °C versus 7 °C (summer and autumn, respectively) to separate the effects of daylength and temperature on photosynthesis and respiration to distinguish which processes are regulated by temperature or daylength only or by a combination of these abiotic factors. In particular, we focused on the effects of these treatments on the acclimation of photosynthetic capacity and energy dissipation and the composition of the photosynthetic apparatus.

Results

Gas Exchange

Needle level gas exchange was performed on samples of seedlings of all four treatments (Fig. 10). At 1,000 μmol photons $m^{-2} s^{-1}$ we observed the highest rate of light-saturated net assimilation (A_{sat}) in the summer conditions with 16 h photoperiod and 22 °C (LD/HT) treatment. In the natural autumn control with 8 h photoperiod/7 °C (SD/LT), A_{sat} was decreased by 19 %. Within the other two treatments, A_{sat} was considerably lower, with the warm autumn conditions with 8 h photoperiod/22 °C treatment (SD/HT) showing 41 % lower values than LD/HT and 28 % lower values than SD/LT. This pattern was also observed when net assimilation was measured at growth light conditions (Fig. 10). While assimilation did not show any clear effect of either temperature or photoperiod and only showed a significant difference in the interaction of both factors, we observed a clear response of respiration to either factor plus the interaction of both factors. Within the warm temperature treatments, the respiration rate in LD/HT was more than twice the rate observed in SD/HT (Fig. 10), suggesting an effect of the shorter photoperiod. Low temperature imposed an additional effect on dark respiration, being responsible for a further decrease in cold summer conditions with 16 h photoperiod/7 °C (LD/LT) and SD/LT needles. These results are also valid when data are expressed on a fresh weight basis because the ratio of fresh weight to leaf area (grams per meter) only changes minimally between treatments over the duration of the experiment (LD/HT, 369 ± 40; SD/HT, 342 ± 19; LD/LT, 341 ± 21; SD/LT, 366 ± 37).

Chl Fluorescence

Chl a fluorescence was measured simultaneously with gas exchange. Maximum photochemical efficiency of PSII in the dark-adapted state (F_v/F_m) was highest in the LD/HT control (Table 1). The sole effect of a decreased length of the photoperiod resulted in a 10 % decrease of F_v/F_m in SD/HT plants. Low temperature had a much stronger effect on decreasing F_v/F_m in the LD/LT compared to the LD/HT treatment. The effect was additive when combining both factors in the SD/LT treatment (51 % decrease) with no interactive effect between daylength and temperature. In both high temperature treatments the quantum yield of PSII did not differ significantly from each other, but

low temperature decreased the yield to less than one-half in LD/LT and to about one-quarter in SD/LT compared to the LD/HT treatment (Fig. 11A). q_P as a measure of the fraction of open PSII RCs revealed large differences that were temperature dependent but did also depend on the interactive effect of daylength and temperature; e.g. values of the SD/HT treatment were 56% higher than in the summer control LD/HT treatment (Fig. 11B). Using nonphotochemical (q_N) and antenna quenching (q_O), we calculated the ratio of q_O/q_N to indicate the relative component of the q_O from all nonphotochemical processes (Table 1). q_O/q_N values were lowest during the typical LD/HT treatment, whereas under SD/HT conditions or under SD/LT conditions, the respective values were 40% and 38% higher than in LD/HT (Fig. 11C), showing solely a photoperiod dependence.

Effects of Photoperiod and Temperature on the Aggregation State of Chl-Protein Complexes

Nondenaturing SDS-PAGE was used to estimate the amount of Chl-binding protein complexes in the thylakoid membrane. The ratio of monomeric lightharvesting complex of PSII ($LHCII^3$) and dimeric LHCII ($LHCII^2$) to oligomeric LHCII ($LHCII^1$) was determined from gel scans to assess the degree of aggregation of LHCII, with a low ratio indicating a high aggregation state of the LHCIIs (Fig. 12). The bulk of LHCII in all four treatments was found in form of oligomeric $LHCII^1$; only one-fifth to one-third, depending on the treatment, was found as $LHCII^2$ and $LHCII^3$. In LD/HT controls, the ratio of $LHCII^{2+3}$/ $LHCII^1$ was 0.66 ± 0.23 and thus about twice the ratio determined in SD/HT (0.35 ± 0.07), LD/LT (0.33 ± 0.08), and SD/LT (0.29 ± 0.08), which indicates a much higher aggregation state in the latter treatments.

Photosynthetic Pigments

Chl a and Chl b levels differed by less than 10% in the two warm temperature treatments, and Chl b increased significantly in the two cold treatments. Photoperiod had no effect on Chl levels (Fig. 13A). However, because the low temperature increases in Chl b exceeded the slight increases in Chl a we observed a temperature-dependent decrease in Chl a/Chl b (Fig. 13B), which also showed an interactive effect. Total carotenoids per

total Chl was constant among all treatments, except for SD/HT needles, which revealed a significant increase of about 15 % compared to the other three treatments (Fig. 13C). In general, the share of neoxanthin and lutein made up approximately one-half of the carotenoid pool and did not vary much between treatments (Fig. 14). In contrast, the fractions of the remaining carotenoids were more variable, indicated, for example, by an increase in β-carotene and an almost complete absence of zeaxanthin in the SD/HT treatment. In turn, this treatment showed the highest amount of violaxanthin but the least amount of antheraxanthin. As a result, the deepoxidation state was considerably lower in SD/HT, reaching only about one-quarter of the values obtained in the other treatments (Fig. 13D). This effect has been observed in two independent experiments carried out in two separate years.

Changes in Proteins of Photosynthesis and Carbohydrate Metabolism

The acclimation in response to daylength and temperature (Fig. 15) shows the highest levels of RC proteins of PSII and PSI (PsbA and PsaA/B, respectively) under LD/HT conditions compared to decreased levels in cold temperature treatments with lowest values in the LD/LT treatment. However, although the overall amount of RC proteins was variable among the treatments, the ratio of PsaA/B over PsbA remained constant (Fig. 15). PsbS levels decreased by 19 % in response to short photoperiod and by 15 % in response to low temperature as compared to the summer control. The additive effect of decreased photoperiod and low temperature resulted in PsbS levels in the SD/LT treatment to be 34 % lower than in LD/HT, indicating that its regulation is independent with no interactive effect of temperature and daylength (Fig. 15).

There was also a clear response of the LHC proteins Lhca1, Lhca4, and Lhcb5 to daylength (Fig. 15). Protein levels in LD/HT and LD/LT clearly exceeded the levels in SD/HT and SD/LT, while there was no effect of temperature. By contrast, Lhcb1 was significantly affected by temperature, as indicated by the accumulation of this protein, with LD/LT being 35 % and SD/LT being 26 % higher than LD/HT and SD/HT levels (Fig. 15). Lhca2 and Lhcb2 exhibited minimal differences between LD/HT and the three other treatments (Fig. 15). The accumulation of the large subunit of Rubisco (RbcL) followed the same pattern observed for the RC proteins of PSII and PSI. Cy-

tosolic Fru-1,6-bisphosphatase (cFBPase) and UDP-Glc pyrophosphorylase (UGPase), both cytosolic enzymes that are required to convert triose phosphate exported from the chloroplast into Suc, were clearly affected by daylength and UGPase also by temperature. Short-day conditions alone induced the accumulation of cFBPase by 27 % and exposure to low temperature by 13 %. A similar pattern was observed in the expression of UGPase (Fig. 15).

Discussion

Short Photoperiod and Low Temperature Promote Down-Regulation of Photosynthetic Capacity

It is remarkable that net CO_2 assimilation in the SD/HT treatment is considerably lower than in the LD/HT and comparable to that of the two low temperature treatments (Fig. 10). Photosynthesis therefore appears to be not only temperature dependent but is also strongly influenced by the length of the photoperiod. In conifers, shortened daylength acts as a signal for the induction of terminal budset and the cessation of growth (Repo *et al.*, 2001). In combination with low temperature, this is an early autumn prerequisite to induce freezing resistance and therefore an important mechanism to prepare for the harsh winter conditions in northern forest environments (Bigras *et al.*, 2001). This actual effect of daylength on photosynthesis probably reflects feedback regulation through an overall decrease in metabolic activity and growth. Such down-regulation of photosynthesis due to a reduced sink capacity is associated with the cessation of growth (Savitch *et al.*, 2000). A decreased rate of CO_2 uptake is therefore likely due to the cessation of growth in pine after cold acclimation. Carbohydrate partitioning undergoes a tremendous shift as growth ceases and metabolic activity decreases under short photoperiod and low temperature. Increased expression of cFBPase, one of the key regulatory enzymes controlling the conversion of triose phosphate exported from the chloroplast into Suc (Strand *et al.*, 2000), is associatedwith short photoperiod (Fig. 15) and indicates increased capacity for Suc synthesis. There is a dual role for Suc during autumn; it can be exported to the roots to be used for starch synthesis for winter storage and it may serve as a cryoprotectant to increase frost resistance in needle tissue. This clearly shows that the regulation of an important pathway for

the cold hardening process strictly requires proper phasing of low temperature and daylength. However, levels of UGPase, acting downstream of cFBPase in the Suc synthesis pathway (Kleczkowski *et al.*, 2004), did not show this exclusive increase due to short daylength but increased also in response to temperature (Fig. 15).

Down-regulation of metabolic processes in response to low temperature and short photoperiod was also reflected in decreased rates of dark respiration in SD/HT, LD/LT, and SD/LT compared to LD/HT. The response of plants to a shorter daylength was a more than 50% decrease in the rate of respiration. However, as a result of warmer temperatures, the respiration rate in the SD/HT treatment remained higher than in the SD/LT treatment.

Acclimation of Photosynthetic Energy Conversion and Composition of the Photosynthetic Apparatus to Growth Conditions

Decreased sink capacity requires acclimation of the energy partitioning process to balance the flow of energy between energy captured by the light reactions and the energy utilized metabolically. This may be achieved by changing properties of the thylakoid membrane-bound LHC proteins, thereby altering the efficiency of capture, conversion, and dissipation of light energy. Several of the LHC proteins showed a response to photoperiod. Lhca1, Lhca4, and Lhcb5 reached the lowest levels in the SD/HT treatment (Fig. 15). This coincided with a low deepoxidation state of the xanthophyll cycle pigments and a lack of zeaxanthin (Figs. 13 and 14), pigments that are preferentially bound in bulk by these proteins (Morosinotto *et al.*, 2002). The down-regulation of photosynthesis in autumn was reflected in partial losses of PSII and PSI RCs as well as the RbcL content, which clearly reflects adjustment of photosynthesis to the reduced need for photosynthates under cold temperatures (see also Zarter *et al.*, 2006). However, in the SD/HT treatment, the decrease of PsbA was balanced by an increase in the fraction of open RCs of PSII, resulting in a similar yield as in LD/HT (Figs. 11, A and B, and 15). While in LD/HT the photosynthetic apparatus remains fully functional and associated with a considerable sink for photosynthates, in low temperature treatments a reduced sink capacity was clearly accompanied by a decrease in the photochemical efficiency. A decrease in photochemical efficiency was not apparent in SD/HT plants, reflecting an

energetic imbalance in these plants. Apparently this imbalance was not reflected by q_P (Fig. 11B), suggesting alternative sinks for electrons that could include photorespiration, water-water cycle, and cyclic electron transport. Increased photorespiration has previously been shown to be an effective photoprotective strategy in high mountain plants under low temperature (Streb *et al.*, 1998). Ivanov *et al.* (2001) suggested that cyclic electron transport around PSI is necessary to dissipate excess energy and to retain functionality of the photosynthetic apparatus in winter-stressed pine needles. Our observation of a significant increase in β-carotene in SD/HT (Fig. 14) points toward an increased formation of singlet-excited oxygen ($^1O_2^*$), which can efficiently be quenched by β-carotene (Cantrell *et al.*, 2003; Krieger-Liszkay, 2005; Telfer, 2005). $^1O_2^*$ is generated from singlet-excited Chl via the Chl triplet state, if the energy of excited Chl cannot be used for photochemistry or dissipated as heat (Adams *et al.*, 2004). There are at least two possible sites of an increased production of $^1O_2^*$ (Krieger-Liszkay, 2005). It could either originate from the antenna, where singlet Chl cannot be quenched due to low concentrations of zeaxanthin and hence passes its energy down to produce $^1O_2^*$, or in the RC of PSII via charge recombination. Here, β-carotene is the principal quencher of excess energy because triplet Chl cannot be quenched directly. In this case, charge recombination would contribute to filling the gap between electrons generated, as measured by the yield and electrons used by CO_2 fixation in the SD/HT treatment.

Dissipation of Excess Energy Depends on the Aggregation State of LHCII

Not only did the yield of photochemistry differ between treatments but we also observed different strategies to dissipate energy that is in excess to be used for photochemistry. It has been suggested that high q_O in relation to q_N is an indicator for high q_O (Bukhov *et al.*, 2001; Sane *et al.*, 2003). We found q_O/q_N values in the LD/HT treatment considerably lower than in the other treatments (Fig. 11C). Previous work also showed that a higher amount of aggregated LHCs is associated with improved photoprotection in overwintering evergreens and other plants (Horton *et al.*, 1991; Ruban *et al.*, 1993; Ottander *et al.*, 1995; Gilmore and Ball, 2000; Krol *et al.*, 2002). Reorganization of pigment-protein complexes, which is indicated by an increased aggregation state of LHCII, was observed in all but the LD/HT control treatment (Fig. 12) and typically coincides with

higher q_O in these treatments. Short photoperiod, as well as low temperature, seem to provide a separate signal that invokes elements of the cold acclimation pathway, resulting in a structural reorganization of the photosynthetic apparatus. Through a constitutive and nonregulated quenching component facilitated by aggregated antenna complexes, SD/HT plants that have been grown under the same temperature regime as the LD/HT plants do not require to accumulate large quantities of zeaxanthin and hence show a much lower deepoxidation of the xanthophyll cycle pigments. Based on these observations, we conclude that zeaxanthin is not the main component required to facilitate the quenching of excess energy in the SD/HT treatment.

Our observations support the LHCII conformation model for NPQ proposed by Horton *et al.* (2005). In this model, the amount of quenching is regulated within minutes by the aggregation state of LHCII in combination with binding of either violaxanthin or zeaxanthin, with the highest quenching efficiency resulting from an aggregated configuration binding zeaxanthin. A similar mechanism might be responsible for the acclimation to seasonal changes in photoperiod and temperature (Fig. 16), with the zeaxanthin-binding aggregated state representing the q_I component of NPQ (Fig. 16C).

Does Increased Autumn Air Temperature Increase Photosynthetic Gain?

Our results indicate an experimentally extended growing season does not necessarily result in increased CO_2 uptake and carbon gain in an evergreen conifer. On the contrary, short-day photoperiod and warm temperatures might even have the opposite effect due to increased rates of respiration and decreased maximum capacity for carbon uptake. Based on these experiments using seedlings in climate controlled chambers, we cannot predict how short photoperiod and warm autumn temperature might affect entire boreal forests in the future. In addition, in our treatments we used a relatively large temperature difference in the SD/LT (7 °C/5 °C) versus the SD/HT (22 °C/18 °C, warm autumn) treatment compared to the predicted increase of mean annual land air surface temperature that is in the range of 4 °C to 6 °C by the end of 2100 (IPCC, 2001). Nonetheless, our results suggest that increased autumn air temperature has the potential to interrupt the regulation of the seasonal development in conifer trees. One can now speculate that there is a temperature within the range of predicted climate

change at which field-grown trees cannot behave optimally and thus cannot exploit the growing season optimally. The temperature increase apparently alters the phasing of the two critical environmental stimuli, thereby not only decreasing the sink capacity of the trees but even turning it into a potential source for the respiratory release of CO_2. Thus, photosynthetic down-regulation due to photoperiodic control of growth cessation during the autumn appears to offset any potential carbon gain resulting from a prolonged growing season in the autumn (Saxe *et al.*, 2001). In response to experimentally increased autumn temperature, Jack pine seedlings exhibit an energy-quenching mechanism that does not follow the well-described PsbS- and zeaxanthin-dependent dissipation pathways. Thus, further investigations are essential to determine whether our findings from controlled environment experiments are consistent with climatic changes under natural environmental conditions on an ecosystem scale.

Materials and Methods

Plant Material and Growth Conditions

Rooted Jack pine (*Pinus banksiana* Lamb.) seedlings were obtained from a local nursery (Somerville Seedlings) and planted in a mixture of ProMix (Premier Horticulture) and low nutrient mineral sand (1:2, v/v). The plants were kept outside underneath a light shelter for 1 year. In the second year, plants were transferred to controlled experimental summer conditions at the end of July, 2005 (Conviron growth chambers). Following 10 d at experimental summer conditions (see below) to allow for acclimation to chamber conditions, plants were exposed for 4 weeks to either 22 °C/18 °C (day/night) with a photoperiod of 16 h (LD/HT), 22 °C/18 °C with an 8 h photoperiod (SD/HT), 7 °C/5 °C with a 16 h photoperiod (LD/LT), or 7 °C/5 °C with an 8 h photoperiod (SD/LT). All treatments were provided with a photosynthetic photon flux density (PPFD) of 350 μmol photons m^{-2} s^{-1}.

Photosynthetic Gas Exchange

CO_2 exchange rates were measured on detached current year needles using a LiCor 6262 infrared gas analyzer connected to a modified LD2/3 cuvette (Hansatech). The needles were collected right before the measurement in the early afternoon, after seedlings had been exposed to growth light of 350 μmol photons m^{-2} s^{-1} for at least 4 h. The

CO_2 concentration was maintained at 375 ppm in air with 21% O_2. Dark respiration was measured in these needles after 20 min of dark adaptation. Subsequently, plants were exposed to a PPFD of 350 μmol photons $m^{-2} s^{-1}$ for 7 min to obtain measurements of steady-state photosynthesis, followed by a shift to a saturating PPFD of 1,000 μmol photons $m^{-2} s^{-1}$ for another 7 min. Steady-state photosynthesis was usually attained within 3 to 6 min, depending on actinic light intensity and temperature. Gas exchange rates are averages over a measuring period of 30 s. All gas exchange measurements were performed at growth temperature.

Chl Fluorescence

Chl a fluorescence was measured with a PAM 2100 Chl fluorometer (Heinz Walz). The fiber optic of the PAM 2100 was connected to the LD2/3 Hansatech cuvette via a custom-made port to allow simultaneous fluorescence and gas exchange measurements after seedlings had been exposed to growth light of 350 μmol photons $m^{-2} s^{-1}$ for at least 4 h (see above). Initial (minimum) PSII fluorescence in the dark-adapted state (F_0) and F_m were determined after 20 min of dark adaptation in the cuvette. F_0', F_m', and transient fluorescence (F_t) were obtained concomitantly with the gas exchange measurements after steady-state photosynthesis was achieved (Sveshnikov *et al.*, 2006). Optimum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$ and the quantum yield of PSII in the light as $F_v'/F_m' = (F_m' - F_t)/F_m'$ (Genty *et al.*, 1989). The fraction of PSII RCs in an open state was estimated as $q_P = (F_m' - F_t)/(F_m' - F_0')$ (Schreiber and Bilger, 1987). Nonphotochemical and antenna quenching were calculated as $q_N = 1 - (F_m' - F_0')/(F_m - F_0)$ and $q_O = 1 - F_0'/F_0$, according to Rees *et al.* (1990).

Isolation of Thylakoid Membranes and Separation of Chl-Protein Complexes

Thylakoids of fresh needles were isolated at 4 °C according to Krol *et al.* (2002) by grinding needles in 50 mM Tricine, pH 7.8, containing 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, and 20% (w/v) polyethylene glycol 4000. The ground samples were filtered through Miracloth, followed by three washing steps in double-distilled water, 1 mM EDTA, and washing buffer, containing 50 mM Tricine, pH 7.8, 10 mM NaCl, and 5 mM $MgCl_2$ by centrifugation at 4 °C at 5,000 g for 10 min. The pellets were solubilized in

300 mM Tricine, pH 8.8, containing 13 % (v/v) glycerol, 0.1 % (w/v) SDS, and 0.45 % (w/v) dodecylmaltoside to give a SDS + dodecylmaltoside:Chl ratio of 20:1 (w/w). The Chl-protein complexes were separated in the dark at 4 °C in nondenaturing 1.3 M Tris-HCl polyacrylamide gels with a Tris/Gly running buffer that contained 0.2 % (w/v) Deriphat 160. The gels were scanned at 671 nm and the relative amount of Chl in each complex was determined as ratio of the peak area to the total area.

Photosynthetic Pigments

Needle samples for the pigment extraction were taken around noon after seedlings had been exposed for 4 h to growth light of 350 μmol photons $m^{-2} s^{-1}$. Needles were ground to a fine powder in liquid nitrogen and extracted for 2 h in the dark on ice in 100 % acetone buffered with $NaHCO_3$. Extracts were separated by HPLC with a Spherisorb ODS-1 analytical column (S.P.E.), modified from Gilmore and Yamamoto (1991) as described in detail by Krol *et al.* (2002). Total Chl and total carotenoids were estimated spectrophotometrically according to Lichtenthaler (1987). The deepoxidation state was calculated as $(0.5\,A + Z)/(V + A + Z)$, where V is violaxanthin, A is antheraxanthin, and Z is zeaxanthin.

Protein Extraction, SDS-PAGE, and Immunoblotting

For protein extraction, needles were ground to a fine powder in liquid nitrogen. Forty milligrams of sample were extracted in 800 μL of ice-cold extraction buffer for 15 min on ice followed by 15 min of extraction at room temperature. The extraction buffer consisted of 60 mM Tris-HCl, pH 6.8, containing 4 % (w/v) SDS, 15 % (w/v) Suc, 20 mM dithiothreitol, and Complete, EDTA-free, proteinase inhibitor cocktail (Roche Diagnostics). Membrane proteins were solubilized for 5 min at 75 °C, cooled on ice for 1 min, and then briefly centrifuged to remove debris from the supernatant. The total concentration of extracted protein was determined after Lowry *et al.* (1951) using the RC DC protein assay kit from Bio-Rad. Protein (7 μg/lane) was loaded and separated electrophoretically at 200 V for 30 min on 10 % (w/v) BisTris gels (Nupage, Invitrogen) using the XCell Midi gel system and a MES/ SDS buffer system (Invitrogen). Following separation, proteins were transferred to a nitrocellulose membrane (0.2 μm pore size, Bio-Rad)

and probed with antibodies against PsbA, PsbS, RbcL, cFBPase, UGPase, the LHC proteins Lhca1, Lhca2, Lhca4, Lhcb1, Lhcb2, and Lhcb5 (Agrisera) as well as against PSI. Goat anti-rabbit and rabbit anti-chicken IgG conjugated with horseradish peroxidase (Sigma-Aldrich) were used as secondary antibodies to allow for chemiluminescent detection of the proteins (ECL detection kit, Amersham) bound to the membrane exposed to x-ray film (Biomax Light, Eastman Kodak). The optical density of each band on the film was quantified using the Scion software package.

Statistics

The effects of daylength and temperature on photosynthetic properties were statistically analyzed by two-way ANOVA at $P < 0.05$ using SPSS version 14.0. All significant differences mentioned in the text refer to the two-way ANOVA results.

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Tables and Figures

Table 1: *The effect of daylength and temperature on Chl fluorescence parameters F_v/F_m, q_O, and q_N, measured under saturating light conditions (1,000 μmol photons $m^{-2} s^{-1}$); n = 8 ± SE. Two-way ANOVA analysis indicates statistically significant differences due to daylength, temperature, or an interactive effect of both factors. ●, ■, and ⋆, Significant differences due to daylength, temperature, and their interactive effect, respectively. One symbol, P ≤ 0.05; two symbols, P ≤ 0.01; three symbols, P ≤ 0.001; n.s., not significant.*

	Treatment				Significance		Interactive Effect
	LD/HT	SD/HT	LD/LT	SD/LT	Daylength	Temp	
F_v/F_m	0.80 ± 0.01	0.72 ± 0.02	0.51 ± 0.05	0.39 ± 0.02	●●●	■■■	n.s.
q_O	0.36 ± 0.03	0.52 ± 0.02	0.35 ± 0.05	0.38 ± 0.03	●●	■	*
q_N	0.93 ± 0.01	0.96 ± 0.01	0.74 ± 0.06	0.71 ± 0.05	n.s.	■■■	n.s.

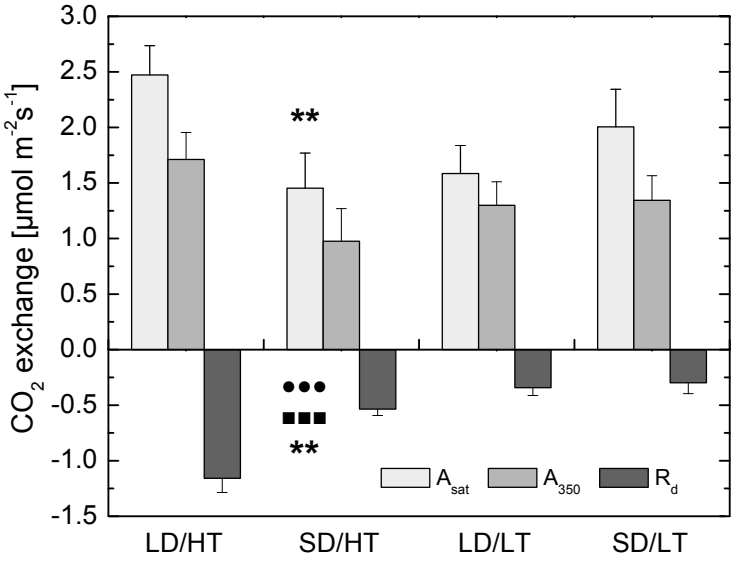


Figure 10: *The effect of daylength and temperature on needle level* CO_2 *assimilation and respiration per needle area. Bars indicate light-saturated net* CO_2 *assimilation, measured at 1,000* μmol photons $m^{-2}\,s^{-1}$ *PPFD* (A_{sat})*,* CO_2 *assimilation at growth light conditions of 350* μmol photons $m^{-2}\,s^{-1}$ *PPFD* (A_{350})*. Black bars indicate dark respiration measured after 20 min of dark acclimation. All measurements were performed at growth temperature (22°C in LD/HT and SD/HT, 7°C in LD/LTand SD/LT plants). Each bar represents the average of n = 7 to 8* ± SE *biological replicates.* ●*,* ■*, and* ⋆*, Significant differences due to daylength, temperature, and their interactive effect, respectively. Two symbols,* $P \leq 0.01$*; three symbols,* $P \leq 0.001$*.*

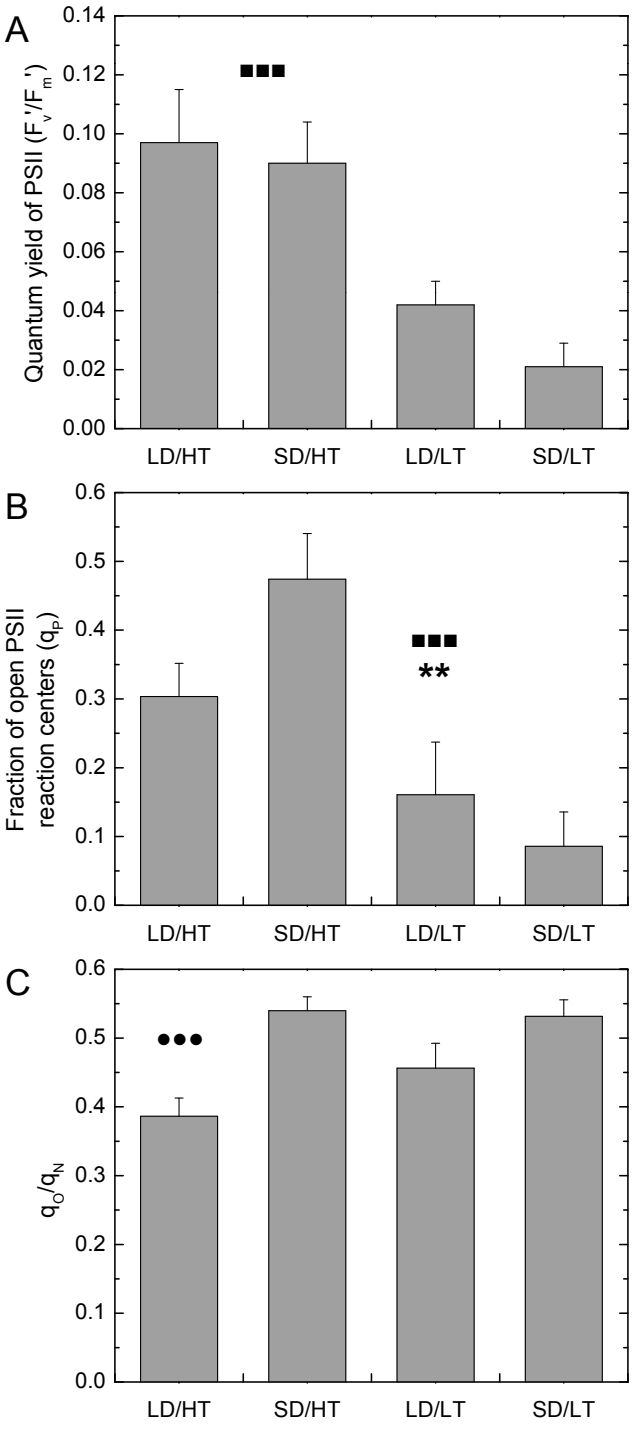


Figure 11: *A, The effect of daylength and temperature on quantum yield of PSII under steady-state condition at 1,000* μmol photons $m^{-2} s^{-1}$ *PPFD. B, Estimated* q_P. *C,* q_O/q_N *as an estimate of the amount of* q_O. *All measurements were performed at growth temperature (22°C for LD/HT and SD/HT and 7°C for LD/LT and SD/LT). Each bar represents the average of n = 8 ± SE biological replicates. ●, ■, and ⋆, Significant differences due to daylength, temperature, and their interactive effect, respectively. Two symbols, P ≤ 0.01; three symbols, P ≤ 0.001.*

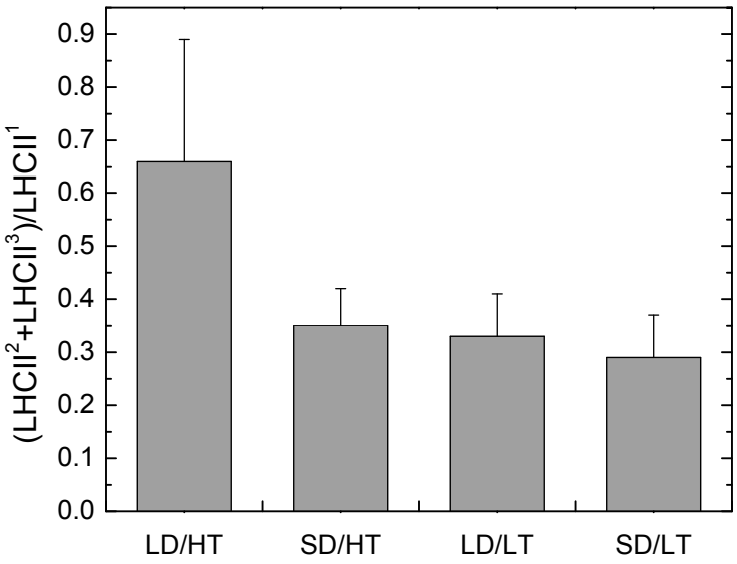


Figure 12: *The effect of daylength and temperature on the degree of aggregation of LHCII. Bars indicate the ratio of LHCII*^{3} *and LHCII*^{2} *to LHCII*^{1} *as determined from nondenaturing SDS-PAGE. Each bar represents the average of n = 4* ± SE *biological replicates.*

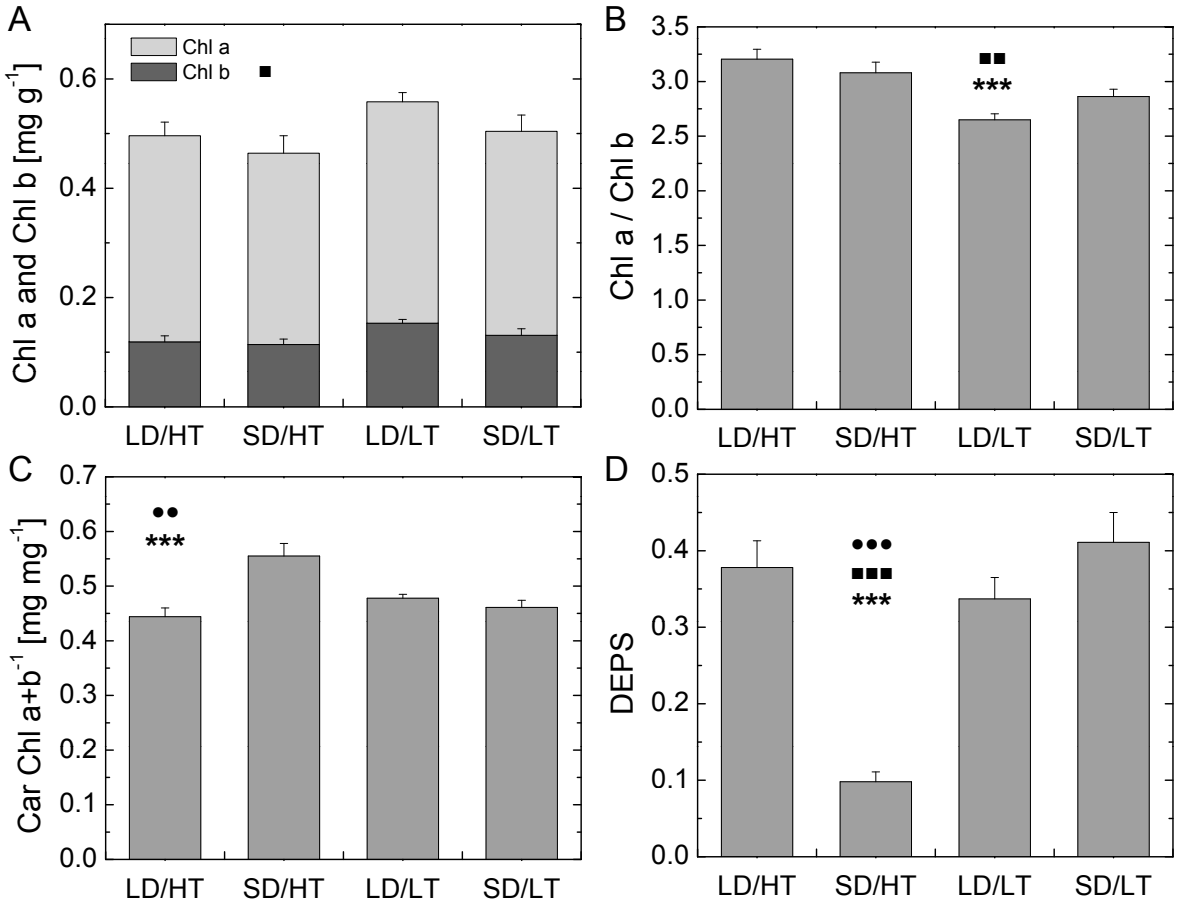


Figure 13: *The effect of daylength and temperature on the composition of photosynthetic pigments in needles of Jack pine. A, Total Chl per fresh weight; Chl b was affected by temperature. B, Chl a to Chl b ratio. C, Total carotenoids per total Chl. D, Deepoxidation status of the xanthophyll cycle pigments, calculated as (0.5 A + Z)/(V + A + Z). Each bar represents the average of n = 8 ± SE biological replicates. ●, ■, and ⋆, Significant differences due to daylength, temperature, and their interactive effect, respectively. One symbol, P ≤ 0.05; two symbols, P ≤ 0.01; three symbols, P ≤ 0.001.*

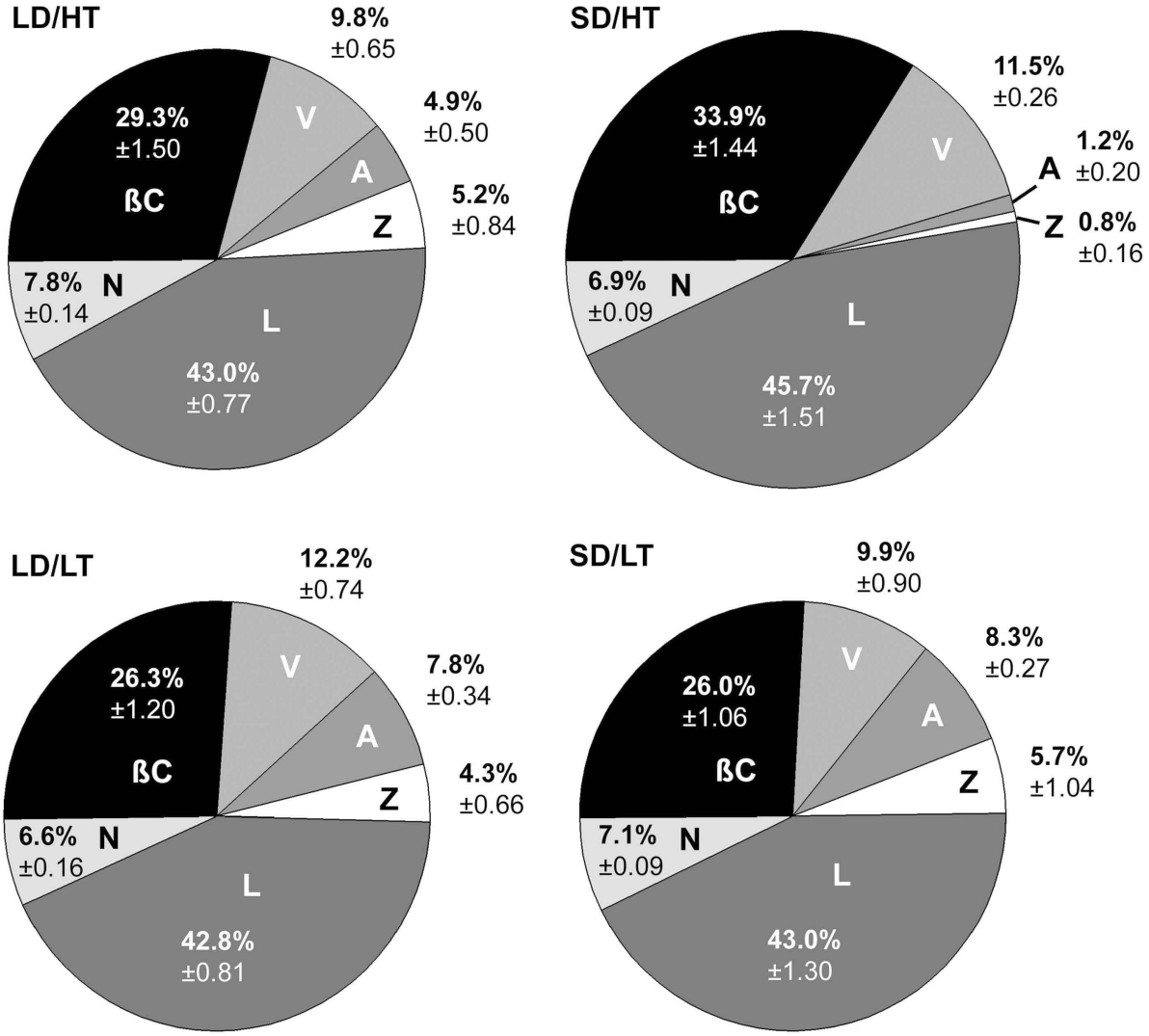


Figure 14: *The effect of daylength and temperature on the carotenoid composition in needles of Jack pine. Each carotenoid component is normalized to the total amount of carotenoids. The relative size of each pie reflects the amount of carotenoids based on a per total Chl basis. βC, β-carotene; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; L, lutein; N, neoxanthin. Values represent the average of n = 8 ± SE biological replicates.*

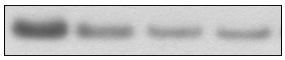
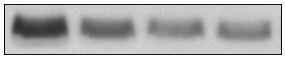
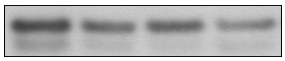
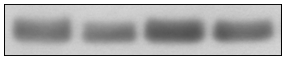
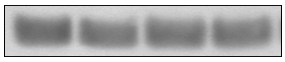
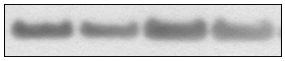
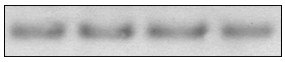
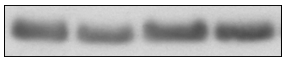
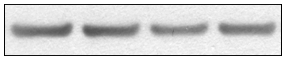
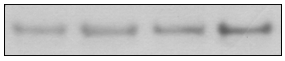
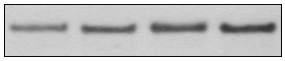
Protein	LD/HT SD/HT LD/LT SD/LT	Treatment LD/HT	SD/HT	LD/LT	SD/LT	Significance dayl.	temp.	inter.
PsbA		1.00 ±0.07	0.68 ±0.05	0.46 ±0.06	0.55 ±0.05	●●	■■■	***
PsaA/B		1.00 ±0.04	0.75 ±0.03	0.44 ±0.05	0.56 ±0.06	n.s.	■■■	***
PsbS		1.00 ±0.04	0.81 ±0.06	0.85 ±0.07	0.66 ±0.05	●●●	■■	n.s.
	ratio PsbA to PsaA/B	1.00 ±0.06	0.90 ±0.07	1.11 ±0.17	1.08 ±0.17	n.s.	n.s.	n.s.
Lhcb1		1.00 ±0.09	1.02 ±0.04	1.35 ±0.02	1.26 ±0.07	n.s.	■■■	n.s.
Lhcb2		1.00 ±0.06	1.14 ±0.06	1.12 ±0.06	1.02 ±0.04	n.s.	n.s.	*
Lhcb5		1.00 ±0.07	0.71 ±0.07	1.02 ±0.05	0.89 ±0.03	●●●	n.s.	n.s.
Lhca1		1.00 ±0.05	0.84 ±0.06	1.10 ±0.06	0.98 ±0.05	●	■	n.s.
Lhca2		1.00 ±0.09	0.96 ±0.11	0.95 ±0.08	0.88 ±0.08	n.s.	n.s.	n.s.
Lhca4		1.00 ±0.08	0.85 ±0.05	1.06 ±0.04	0.99 ±0.04	●	n.s.	n.s.
RbcL		1.00 ±0.03	0.86 ±0.02	0.75 ±0.03	0.81 ±0.03	n.s.	■■■	***
cFBPase		1.00 ±0.07	1.27 ±0.12	1.13 ±0.11	1.47 ±0.18	●	n.s.	n.s.
UGPase		1.00 ±0.09	1.33 ±0.11	1.35 ±0.10	1.54 ±0.13	●	■	n.s.

Figure 15: *The effect of daylength and temperature on the expression levels of key proteins of photosynthesis in needles of Jack pine. The average optical density of the LD/HT treatment was arbitrarily scaled to 1. Typical bands from the original western blots are shown next to the values, with each lane loaded on an equal protein basis. Each value represents the average of n = 8 ± SE biological replicates. Two-way ANOVA analysis indicates statistically significant differences due to daylength, temperature, or an interactive effect of both factors.* ●, ■, *and* ⋆, *Significant differences due to daylength, temperature, and their interactive effect, respectively. One symbol, P ≤ 0.05; two symbols, P ≤ 0.01; three symbols, P ≤ 0.001; n.s., not significant.*

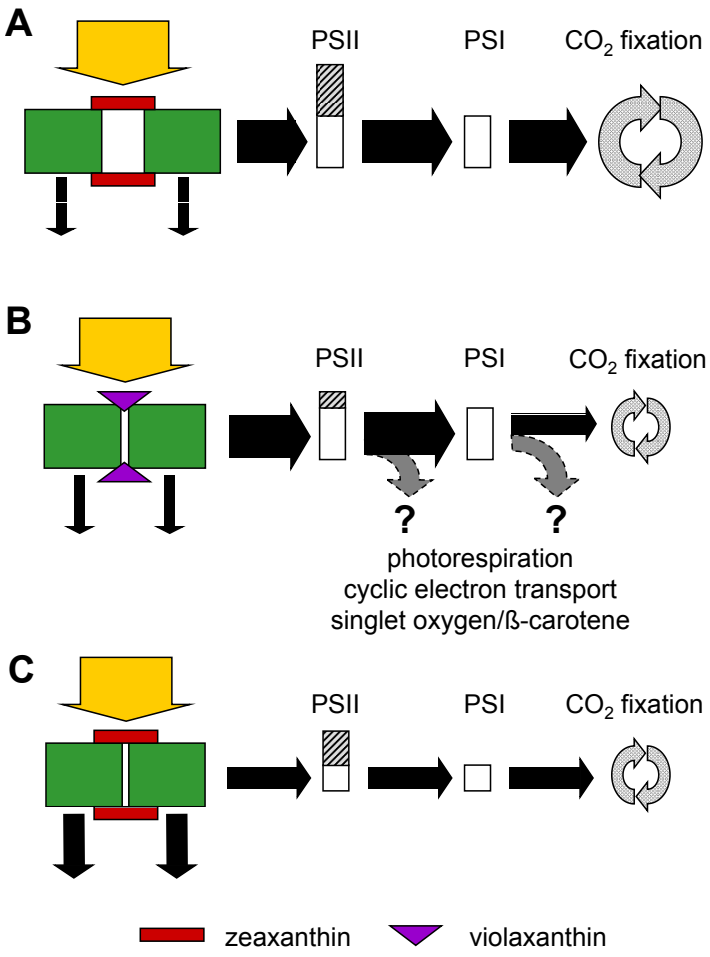


Figure 16: *Model of extended energy quenching including LHCII aggregation. The model depicts the amount of photochemical and NPQ controlled by the deepoxidation state of the xanthophyll cycle and the aggregation state of LHCII, based on the model of Horton et al. (2005). Energy absorbed (yellow arrows) is quenched either nonphotochemically (black arrows) in the antenna complex (green rectangles) or photochemically through the photosystems (white parts, open RCs; hatched parts, closed RCs) and used for CO_2 fixation (gray arrows). Depending on the aggregation state of LHCII (represented by the proximity of the green rectangles) and the xanthophyll configuration, energy is preferentially quenched one way or the other (the thickness of the arrows marks the efficiency of the respective processes). State A refers to the situation found in our LD/HT treatment, with a low aggregation state of LHCII in combination with a high deepoxidation state. State B is dominant in the SD/HT treatment, where high aggregation of LHCII coincides with a very low zeaxanthin concentration. This results in nonphotochemical and photochemical quenching that compares to the situation observed in state A, except that not all of the energy provided through the photochemical quenching process is used for CO_2 fixation. In this situation, alternative electron sinks, including photorespiration, water-water cycle, and charge recombination, play a prominent photoprotective role to support the photochemical quenching process. State C refers to both the LD/LT and SD/LT treatments, with high aggregation and high deepoxidation states; a large portion of the incident light is quenched in the antenna, photochemical quenching is relatively small. See text for further details.*

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III

III

Increased Air Temperature during Simulated Autumn Conditions Impairs Photosynthetic Electron Transport Between PSII and PSI

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Abstract

Changes in temperature and daylength trigger physiological and seasonal developmental processes that enable evergreen trees of the boreal forest to withstand severe winter conditions. Climate change is expected to increase the autumn air temperature in the northern latitudes while the natural decreasing photoperiod remains unaffected. As shown previously, an increase in autumn air temperature inhibits CO_2 assimilation with a concomitant increased capacity for zeaxanthin-independent dissipation of energy exceeding the photochemical capacity in *Pinus banksiana*. In this study we tested our previous model of antenna quenching and tested a limitation in intersystem electron transport in plants exposed to elevated autumn air temperatures. Using a factorial design, we dissected the effects of temperature and photoperiod on the function as well as the stoichiometry of the major components of the photosynthetic electron transport chain in *P. banksiana*. Natural summer conditions (LD/HT: 16 h photoperiod/22 °C)

and late autumn conditions (SD/LT: 8 h/7 °C) were compared to a treatment of autumn photoperiod with increased air temperature (SD/HT: 8 h/22 °C) and a treatment with summer photoperiod and autumn temperature (LD/LT: 16 h/7 °C). Exposure to SD/HT resulted in an inhibition of the effective quantum yield associated with a decreased PSII/PSI stoichiometry coupled with decreased levels of Rubisco. Our data indicate that a greater capacity to keep the photoactive chlorophyll a molecule of photosystem I (P700) oxidized in plants exposed to SD/HT compared to the summer control may be attributed to a reduced rate of electron transport from the cytochrome b_6f complex to PSI. Photoprotection under increased autumn air temperature conditions appears to be consistent with zeaxanthin-independent antenna quenching through light-harvesting complex II aggregation and a decreased efficiency in energy transfer from the antenna to the PSII core. We suggest that models that predict the effect of climate change on the productivity of boreal forests must take into account the interactive effects of photoperiod and elevated temperatures.

Introduction

Cold hardening in conifers is a physiological process which includes the cessation of growth and long-term changes in metabolism. In evergreen trees of the boreal forest, this process is triggered by short days and potentiated by low temperature (Weiser, 1970; Christersson, 1978; Bigras *et al.*, 2001; Li *et al.*, 2002; Beck *et al.*, 2004; Puhakainen *et al.*, 2004). One of the major challenges for overwintering evergreen conifers like *Pinus banksiana* is balancing the photosynthetic electron transport rate with the rate of consumption of reductant by metabolism (Busch *et al.*, 2007). This is especially important since evergreen conifers retain a substantial amount of chlorophyll (Chl) throughout the winter and hence continue to absorb light while at the same time the short photoperiod-, low temperature-induced, down-regulated metabolism is not able to utilize the absorbed energy. Light capture and energy utilization are regulated in a coordinated manner to prevent oxidative damage to the photosynthetic apparatus. Energy balance, defined as photostasis (Öquist and Hüner, 2003), is achieved by reorganization of the photosynthetic machinery, including changes in antenna size and organization, adjustments of protein and Chl concentrations and a range of alternative energy dissi-

pation pathways (Demmig-Adams *et al.*, 1996; Asada, 1999; Öquist and Hüner, 2003; Ensminger *et al.*, 2004; Horton *et al.*, 2005; Ensminger *et al.*, 2006; Sveshnikov *et al.*, 2006; Rumeau *et al.*, 2007).

Under conditions where more light energy is absorbed than can be utilized, an increase in non-radiative dissipation can be observed as non-photochemical quenching of chlorophyll fluorescence (NPQ). NPQ is linked to the deepoxidation of violaxanthin to zeaxanthin via the xanthophyll cycle (Demmig-Adams *et al.*, 1996). It consists of different components that are distinguished by their characteristics in induction and relaxation kinetics (Müller *et al.*, 2001). The major component of NPQ is q_E, which is dependent on the pH gradient across the thylakoid membrane. It builds up and relaxes rapidly within seconds to minutes. A more sustained quenching mode is represented by q_I, which relaxes more slowly and is related to photoinhibition of photosynthesis (Müller *et al.*, 2001).

Not all of the energy absorbed in excess is dissipated in the antenna. Electrons in excess are also used by photorespiration (Wingler *et al.*, 2000) or to produce ATP via cyclic electron flow (CEF) (Rumeau *et al.*, 2007). Excitation pressure (Hüner *et al.*, 1998) can be reduced by transferring electrons to oxygen via the water-water cycle (Asada, 2000) or via chlororespiration through the plastid terminal oxidase (PTOX) (Peltier and Cournac, 2002; Rumeau *et al.*, 2007). Aside from being upregulated under stress conditions (Streb *et al.*, 2005; Quiles, 2006), PTOX is involved in carotenoid biosynthesis (Carol *et al.*, 1999). However, overexpression of PTOX did not result in an increased resistance to photoinhibition in Arabidopsis (*Arabidopsis thaliana*) and therefore it has been suggested that PTOX cannot be considered a significant protective safety valve in fully expanded leaves of Arabidopsis (Rosso *et al.*, 2006). Intersystem electron transport is dependent on the diffusion of plastoquinone (PQ) between PSII and the cytochrome (Cyt) b_6f complex (Haehnel, 1984). Plastocyanin (PC) may also regulate electron flux under conditions where carbon assimilation is limited (Schöttler *et al.*, 2004). It has been demonstrated that only a minor fraction of PC, located in the outer regions of grana, is used to facilitate rapid electron transfer between the Cyt b_6f complex and PSI (Kirchhoff *et al.*, 2004). This electron transfer can be severely inhibited by an

impairment of PC diffusion due to proteins protruding into the luminal diffusion space or a reduction in the volume of the thylakoid lumen (Haehnel, 1984).

The northern latitudes, habitat of tundra and boreal forests, have experienced a dramatic increase in surface air temperature over the past decades, with increases observed especially in the winter and this trend is expected to continue (ACIA, 2005; IPCC, 2007). Given these changes in the arctic climate, the length of the growing season is likely to increase by 20 – 30 days by 2080 (ACIA, 2005) and thereby improve the productivity of northern hemisphere forests (White *et al.*, 2000; Saxe *et al.*, 2001). However, it has recently been shown that boreal evergreen conifers might not be able to fully exploit an extended growing season, as result of either an earlier onset of spring conditions (Slot *et al.*, 2005; Ensminger *et al.*, 2008) or prolonged warmer temperatures during autumn (Busch *et al.*, 2007). Under simulated increased autumn air temperatures in *Pinus banksiana*, carbon assimilation rates were reduced with a concomitant increase in non-photochemical quenching of absorbed energy. However, the energy quenching mechanism was inconsistent with the well-described zeaxanthin- and PsbS-dependent dissipation pathways for photoprotection (Busch *et al.*, 2007). A model was proposed whereby antenna quenching was dependent on the deepoxidation state of the pool of xanthophyll cycle pigments as well as on protonation of LHCII, affecting the degree of LHCII aggregation (Horton *et al.*, 2005; Busch *et al.*, 2007). To test this model, we assessed the effects of elevated autumn air temperatures in *P. banksiana* on photosystem stoichiometry, antenna quenching, xanthophyll cycle activity and intersystem electron transport. As controls we used two treatments representing, first, natural summer conditions (LD/HT: 16 h photoperiod/22 °C), and second, late autumn conditions (SD/LT: 8 h/7 °C). To simulate increased autumn air temperatures, a treatment with autumn photoperiod and summer temperature (SD/HT: 8 h/22 °C) was compared to the two controls. In addition, a second experimental treatment with summer photoperiod and autumn temperature (LD/LT: 16 h/7 °C) was used to separate the effects of photoperiod and temperature.

Results

Effects of Photoperiod and Temperature on the Polypeptide Composition of the Photosynthetic Apparatus

Regardless of photoperiod, Lhcb1 levels in LD/HT and SD/HT plants were about 50% lower than in plants exposed to either LD/LT or SD/LT (Fig. 17A) which is consistent with previous results (Busch *et al.*, 2007). The abundance of the PSII reaction center (RC) polypeptide, PsbA, was highest in the summer control (LD/HT) and decreased to 20±4% of that amount in the autumn control (SD/LT) (Fig. 17B). The amount of PsbA in *P. banksiana* exposed to SD/HT conditions was intermediate to that of the two control treatments (65±9% of LD/HT) and attained the lowest levels in LD/LT plants (9±3%). The RC polypeptides of PSI (PsaA/B) also reached the highest amounts in LD/HT plants and the levels in SD/LT plants were less than half of that (45±9%). In plants exposed to SD/HT, however, PsaA/B did not decrease and the amount was comparable to the summer control (LD/HT) (108±7%). As for PsbA, the lowest amounts of PsaA/B were also detected in LD/LT plants (18±2%) (Fig. 17C). Cyt *f*, a protein linking the electron transport chain between PSII and PSI, and RbcL, the large subunit of the Calvin cycle protein Rubisco, followed a similar pattern as PsbA. For Cyt *f*, the highest amounts were detected in the summer control (LD/HT) and decreased to 90±17% in the autumn control (SD/LT) (Fig. 17D). In SD/HT plants the amount of Cyt *f* was 85±15% of LD/HT plants and the lowest amount again was detected in LD/LT plants (30±8%). The amount of PC, which transfers electrons from the Cyt b_6f complex to PSI, was comparable between the two control treatments with the autumn control (SD/LT) reaching 91±11% of the summer control (LD/HT) (Fig. 17E). In contrast to the other components of the photosynthetic apparatus, the amount of PC increased to 115±11% in SD/HT plants as compared to the summer control (LD/HT). The lowest amounts were detected in LD/LT plants (73±14%). RbcL in the autumn control (SD/LT) (61±6%) was significantly reduced as compared to the summer control (LD/HT) (Fig. 17F). In plants exposed to SD/HT, the amount of RbcL (74±7%) was intermediate between the summer and the autumn controls and the lowest amounts were observed under LD/LT conditions (51±6%). PTOX responded to exposure to the autumn control conditions (SD/LT) by increasing protein content to 276±20% relative to the summer control (LD/HT). Sim-

ilar increases were detected for plants exposed to LD/LT conditions (289±17%). However, when *P. banksiana* was treated to SD/HT conditions, PTOX content (343±24%) was stimulated to the greatest extent (Fig. 17G).

Effects of Photoperiod and Temperature on Photosynthetic Pigments

In order to test the validity of the quenching mechanism in the model proposed by Busch *et al.* (2007), we tested the activity of the xanthophyll cycle. Fig. 18A shows the deepoxidation status (DEPS) of the xanthophyll cycle pigments for the four treatments in response to short-term (2 h) exposure to various light intensities. All treatments show a saturation of DEPS at light intensities of 800 μmol photons m^{-2} s^{-1} or more. Major differences between the four treatments can be seen in the dark adapted state and at light intensities ranging up to 500 μmol photons m^{-2} s^{-1}. In the summer control (Fig. 18A, closed circles), DEPS was considerably lower than in the autumn control (Fig. 18A, open squares). The highest DEPS at all light intensities was detected in plants exposed to LD/LT conditions (Fig. 18A, open squares), which reflects the fact that most of the xanthophyll cycle pigments were found in the form of zeaxanthin. Thus, the results for summer (LD/HT) and autumn controls (SD/LT) as well as plants exposed to LD/LT indicate that *P. banksiana* exhibits a normal xanthophyll cycle. Interestingly, DEPS in plants exposed to SD/HT was substantially lower than even the summer control (Fig. 18A, closed squares), reaching only about one third of LD/HT in the dark adapted state.

The total size of the xanthophyll cycle pigment pool exhibited only minimal changes between treatments and was only dependent on photoperiod, but not on temperature (Fig. 18B). Therefore, decreased levels of DEPS also indicate decreased total amounts of zeaxanthin, as the overall amount of xanthophyll cycle pigments did not increase. In SD/HT and SD/LT exposed plants, the pool size was 13% less than in LD/HT plants. LD/LT plants, which showed the highest DEPS, had 23% more xanthophyll cycle pigments than the summer control and about 40% more than the short day treatments (Fig. 18B).

β-carotene levels were equivalent in the summer and autumn controls (Fig. 18C). However, under SD/HT conditions we observed about 50% higher levels of β-carotene

than in either the summer or autumn control treatments as well as plants exposed to LD/LT conditions.

Effects of Photoperiod and Temperature on PSII and PSI function

PSII function

Chlorophyll fluorescence was used to probe PSII function. In accordance with the abundance of PsbA (Fig. 17B), photochemical efficiency (F_v/F_m) was highest in the summer control (LD/HT: 0.77±0.01). In contrast, the autumn control plants were photoinhibited and showed a severely reduced F_v/F_m (SD/LT: 0.15±0.02), similar to that of plants exposed to LD/LT conditions (0.08±0.01). However, under increased autumn air temperatures, we observed minimal photoinhibition (SD/HT: F_v/F_m = 0.70±0.02). Clearly, a low temperature growth regime had a significant effect on maximum photochemical efficiency whereas the effect of photoperiod appeared to be minimal.

To examine this in more detail, we assessed the effects of growth regime on the fluorescence induction curves. In all four treatments, fluorescence yield (F_s) initially increased when the light was turned on and subsequently decreased to steady state levels (Fig. 19). In the autumn control as well as in plants exposed to LD/LT, changes in the fluorescence yield were minimal and F_s reached F_0 levels within about 1 min. These results are consistent with those of Fv/Fm. Consistent with the F_v/F_m data, both summer control (LD/HT) and SD/HT plants exhibited significant increases in F_s upon exposure to actinic light. However, F_s was rapidly quenched within the first minute to levels that were lower than F_0. The extent of this quenching appeared to be greater in plants exposed to SD/HT conditions than the summer control (LD/HT). This effect was reversible when the light was switched off. After turning off the actinic light, a very rapid recovery of F_s close to F_0 levels within minutes was observed in SD/HT plants, but not in any other treatment. This rate of recovery of F_s was enhanced by applying far-red (FR) light (Fig. 19, Insert).

Since there appeared to be a considerable effect of both growth temperature and photoperiod on fluorescence quenching, we examined the effects of growth regime on the light response curves for fluorescence quenching parameters, the redox state of PSII as well as the effective quantum yield of PSII (Fig. 20). Plants grown at low temperature

exhibited a significantly lower amount of NPQ than plants grown at high temperature irrespective of photoperiod (Fig. 20A). At light intensities up to 800 μmol photons $m^{-2} s^{-1}$, NPQ was slightly higher in SD/HT than in LD/HT plants. Under low to moderate light intensities, NPQ responded stronger to an increase in light intensity in SD/HT than in LD/HT plants (Fig. 20A) and thus showed a 1.55 fold maximum quantum efficiency for NPQ (LD/HT: 8.5±1.5 x10^{-3} NPQ/ μmol photons $m^{-2} s^{-1}$; SD/HT: 13.2±2.4 x10^{-3}), determined as the initial slope of the light saturation curve for NPQ. In plants treated with low temperature, maximum quantum efficiency of NPQ was reduced 6 to 9 fold irrespective of photoperiod (LD/LT: 1.7±0.1 x10^{-3}; SD/LT: 1.5±0.3 x10^{-3} NPQ/ μmol photons $m^{-2} s^{-1}$). These trends were confirmed by assessing antenna quenching measured as q_O (Fig. 20B).

Excitation pressure ($1-q_P$), a measure of the reduction state of PSII reaction centers, was lowest in the summer control (LD/HT) and was highest in the autumn control (SD/LT) and in plants exposed to LD/LT (Fig. 20C). Over the whole range of light intensities tested, SD/HT plants showed a $1-q_P$ higher than the summer control but lower than the autumn control. Thus, although low temperature had the greatest effect on excitation pressure, combining summer temperatures with a short photoperiod significantly increased the proportion of closed PSII reaction centers.

Fig. 20D depicts the effective quantum yield of PSII (F_v'/F_m'). Summer control (LD/HT) plants showed the highest yield across all light intensities. In the autumn control (SD/LT) as well as in LD/LT F_v'/F_m' was substantially decreased. However, by changing only the photoperiod from LD/HT to SD/HT caused a decrease in F_v'/F_m' to about half of the summer control (Fig. 20D).

PSI function

The extent of FR light-induced absorbance change was used to estimate PSI function *in vivo* (Klughammer and Schreiber, 1991; Ivanov *et al.*, 1998). Both the autumn control (SD/LT) and plants exposed to LD/LT exhibited a 30 to 50 % lower relative amount of FR-oxidized $P700^+$, estimated as $\Delta A_{820}/A_{820}$, than the summer control (LD/HT) (Fig. 21A). This is consistent with the levels of PsaA/B detected in these samples (Fig. 17C). However, under SD/HT conditions, we observed an increase of 67 % in

the $\Delta A_{820}/A_{820}$ signal as compared to summer LD/HT conditions, which could not be accounted for by higher levels of PsaA/B (Fig. 17C).

The summer control (LD/HT) showed the shortest halftime for $P700^+$ reduction after the FR light source was turned off (Fig. 21B). The halftime of the autumn control (SD/LT) was 61 % higher, which is an indication of lower PSI cyclic electron transport (Maxwell and Biggins, 1976), as compared to the summer control (LD/HT). Under SD/HT we observed an increase in the halftime of $P700^+$ reduction by 91 % relative to LD/HT. The slowest $P700^+$ reduction was detected in LD/LT with an increase of 182 % as compared to LD/HT. Clearly, exposure to both low temperature as well as a short photoperiod increased the $t_{1/2}$ for dark reduction of $P700^+$ relative to summer control plants.

We found a clear temperature dependency in the pool size of electrons in the intersystem electron transport chain (e^-/P700), calculated as the area ratio of multiple turnover (MT) to single turnover (ST) flash (Fig. 21C). The intersystem electron pool size in the summer control (LD/HT) was about twice as large as that of either the autumn control or plants exposed to LD/LT. Plants exposed to SD/HT conditions exhibited a pool size similar to that of the summer control.

Energy distribution between PSII and PSI

Changes in the PSII and PSI polypeptide content (Fig. 17B,C) were also reflected in the 77 K fluorescence emission spectra (Fig. 22A). Shown are the averages of eight spectra for each treatment for all four treatments. Average spectra illustrate the emission obtained between 650 and 800 nm when excited at 436 nm, normalized to the emission of 685 nm. As expected, fluorescence maxima were detected at 685 nm 694 nm and 731 nm in the summer control (LD/HT, solid line), the first two representing emissions from the PSII core and the last from PSI (Krause and Weis, 1991). Under autumn control conditions (SD/LT), the amplitude of the long wavelength-fluorescence peak increased and the peak maximum was shifted from 731 nm to 723 nm. It is obvious from Fig. 22A that the emission ratio of PSII/PSI strongly decreases in both the autumn control (SD/LT) and LD/LT plants as compared to the summer control (LD/HT), reflecting the ratio in PsbA to PsaA/B polypeptide composition (Fig. 17H). However, in plants exposed to

SD/HT conditions, the lower ratio of PsbA to PsaA/B polypeptides was not reflected in major changes in the ratio of PSII/PSI fluorescence emission.

In addition, the difference spectra between the autumn and summer controls (SD/LT −LD/HT) indicated a distinct peak at 676 nm (Fig. 22B). A similar peak at 676 nm was observed in the difference spectra between plants exposed to LD/LT and the summer control (LD/LT−LD/HT). Thus, exposure of *P. banksiana* to low temperature appears to cause a blue shift in the PSI emission at 731 nm (Fig. 22A) and enhanced fluorescence emission at 676 nm (Fig. 22B). Although exposure of plants to SD/HT caused a minimal blue shift in the PSI emission band at 731 nm, this did result in enhanced emission at 676 nm relative to the summer control as observed in plants exposed to low temperature (Fig. 22B).

Discussion

Exposure to SD/HT conditions alters the structure and composition of the photosynthetic apparatus causing inhibition of photosynthetic electron transport

Pinus banksiana is an important species in boreal evergreen forests. Global climate change measurements over the past decades indicate that the average surface air temperature has increased significantly and this warming trend is predicted to continue (ACIA, 2005; IPCC, 2007). It has been suggested that this would lead to an increase in the growing season, and thus, enhance the productivity of the boreal forests. Changes in temperature and photoperiod are the predominant environmental signals used by conifers such as *P. banksiana* to induce the dormant state in the autumn and achieve cold hardiness, which is required by vegetative tissue and reproductive organs to survive boreal winter conditions. However, the warming trend in the northern latitudes occurs with no change in seasonal photoperiods. Recently, we reported that, contrary to expectations, increased autumn temperature combined with a normal, short autumn photoperiod (SD/HT) inhibits rather than enhances photosynthetic capacity of *P. banksiana* measured as CO_2 assimilation (Busch *et al.*, 2007). Here, we show that this inhibition of photosynthesis in plants exposed to SD/HT can be accounted for, at least in part, by a significant impairment of photosynthetic electron transport due to

alterations in the structure and composition of the photosynthetic apparatus. First, by probing PSII function by Chl a fluorescence, we observed that, relative to our summer control (LD/HT), exposure to SD/HT conditions inhibited the effective quantum yield (Fig. 20D), which was associated with increased excitation pressure, measured as $1-q_P$ (Fig. 20C). Second, by probing the redox state of PSI by measuring $\Delta A_{820}/A_{820}$ (Fig. 21A), we observed that plants exposed to an autumn photoperiod but elevated temperatures (SD/HT) exhibited higher levels of $P700^+$ relative to the summer control (LD/HT). Since this was associated with lower levels of Rubisco but comparable levels of PsaA/B (Fig. 17B,F), the greater capacity to keep P700 oxidized in SD/HT compared to LD/HT plants cannot be due to enhanced photosynthetic capacity since CO_2 assimilation is inhibited (Busch *et al.*, 2007). Although there appears to be a limitation on the acceptor side of PSI based on Rubisco levels (Fig. 17F) and CO_2 assimilation rates (Busch *et al.*, 2007), there must be an even greater limitation on the donor side of PSI to account for this higher capacity to keep P700 oxidized in SD/HT versus LD/HT plants.

Where is the site of limitation in photosynthetic electron transport in plants grown at SD/HT conditions? The site of limitation is not k_1 (Fig. 23), since the quenching in the light (Fig. 19) and considerable $1-q_P$ (Fig. 20C) indicate a buildup of a proton gradient due to PQ reduction, and there were no significant differences in either F_v/F_m or intersystem electron pool size (e^-/P700) (Fig. 21C) in SD/HT plants compared to summer control plants (LD/HT). Assuming Cyt b_6f is evenly distributed throughout grana and stroma (Albertsson, 2001), the diffusion distance for the reduced PQ is kept short and therefore is not likely to pose a major limitation on photosynthetic electron transport. The NAD(P)H dehydrogenase complex appears to be absent in pine chloroplasts (Wakasugi et al., 1994), therefore, it is unlikely that the stroma (k_2) significantly contributes to the PQ reduction. PTOX accumulation does not alleviate excitation pressure under SD/HT conditions, making it unlikely for k_3 to be a major exit route for electrons. The limiting step is also not between PSI and the assimilation of CO_2 (k_5), given that high levels of $P700^+$ are associated with lower levels of Rubisco and reduced CO_2 assimilation rates (Busch *et al.*, 2007). Because cyclic electron transport (k_6) is reduced, we suggest that the probable limitation in photosynthetic electron transport must be

between Cyt b_6f and PSI (k_4) (Fig. 23). This would be consistent with the observation that plants exposed to SD/HT conditions exhibited a greater quenching of F_s, which was rapidly relaxed either in the dark or by the addition of FR light to activate PSI (Fig. 19). Therefore, the rate limiting step in the transfer of electrons from PSII to PSI would be the limitation in diffusion of PC. Diffusion of PC could be impaired by (1) a reduction of the lumen width, e.g. as a result of hyperosmotic stress (Cruz *et al.*, 2001), (2) protruding proteins in the luminal diffusion space (Kirchhoff *et al.*, 2004), or (3) a change in PC concentration or a greater spatial separation of PSII and PSI. Golding and Johnson (2003) proposed a model in which the spatial separation of PSII and PSI is exacerbated under unfavourable conditions due to a shift of active PSI from the grana margins to a separate pool in the stroma thylakoids, which would limit electron transfer between PSII and PSI. A situation where the reduction of Cyt b_6f by PQ occurs faster than its re-oxidation by PC, likely due to slow PC migration, has been reported in ageing tobacco (*Nicotiana tabacum*) leaves (Schöttler *et al.*, 2004).

LHCII trimerization facilitates zeaxanthin independent quenching of excess energy

The xanthophyll cycle generally plays a major role in the photoprotection of plants and thermal dissipation of excess energy under high light is facilitated by zeaxanthin and antheraxanthin in the antenna of PSII (Adams *et al.*, 2004). However, previous work has shown that the zeaxanthin-dependent pathway is not the dominant mechanism to dissipate excess energy in the antenna of SD/HT plants and a model was proposed in which antenna quenching occurs by increased LHCII trimerization (Busch *et al.*, 2007). Here we provide functional evidence that plants exposed to SD/HT conditions, indeed, exhibit enhanced antenna quenching compared to the summer control (LD/HT) when measured as q_O (Fig. 20B). Furthermore, *in vitro*, the transition from monomeric to trimeric LHCII is characterized by an increase in 77 K fluorescence at 675 nm (Klimov, 2003; Wentworth *et al.*, 2004). Our fluorescence difference spectra, obtained by subtracting the LD/HT trace from the traces of each treatment, show a peak at this wavelength, indicating an increase in the proportion of trimeric LHCII (Fig. 20B). This is in agreement with our previous observations based on nondenaturing SDS-PAGE, show-

ing an increased ratio of trimeric to monomeric LHCII (Busch *et al.*, 2007). We also observed a decrease of the peak at 694 nm relative to the peak at 685 nm (Fig. 22), which originates from the PSII core and LHCII, respectively (Vandorssen *et al.*, 1987; Krause and Weis, 1991). These results indicate that the efficiency of energy transfer from the antenna to the core is decreased in SD/HT as compared to LD/HT and especially in the two low temperature treatments. We suggest that, at least in SD/HT plants, this is due to a decrease in the relative amount of the minor LHC, as previously shown for Lhcb5 (Busch *et al.*, 2007), the innermost LHC protein, connecting LHCII to the PSII core (Bossmann *et al.*, 1997). We conclude that the trimerization of LHCII together with the uncoupling from the core may protect PSII from photodamage under conditions where the capacity for CO_2 assimilation is suppressed.

Fig. 18A shows that plants exposed to SD/HT have by far the lowest DEPS, yet the xanthophyll cycle is fully functional in all four treatments. Whereas in the two low temperature treatments the xanthophyll pool is largely deepoxidized even in the dark, the two high temperature treatments exhibit this behaviour only under high irradiance. The lower DEPS in SD/HT as compared to LD/HT plants is not due to a larger total pool size of the xanthophyll cycle pigments, as can be seen in Fig. 18B. Despite the low DEPS, antenna quenching, which should be mediated by zeaxanthin present in the antenna system, was highest in SD/HT (Fig. 20B). Thus, we propose that exposure of plants to SD/HT induces a higher aggregation state of LHCII, which constitutively quenches excess energy through a zeaxanthin-independent mechanism (Busch *et al.*, 2007). We further propose that this zeaxanthin-independent quenching is due to an increased q_E (the fast relaxing component of NPQ), mediated by an increase in the pH gradient due to a reduction in CO_2 assimilation rates. This increase in q_E is associated with an increased quantum efficiency for NPQ formation. Increased q_E in SD/HT plants is also indicated by our observation of a very fast relaxation of steady-state fluorescence back to F_0 levels after the actinic light was turned off (Fig. 19).

In addition, under increasing light intensities, violaxanthin is increasingly converted to zeaxanthin, which provides additional photoprotection via zeaxanthin- dependent NPQ. This mechanism seems to be essential especially for LD/LT, which has the highest DEPS across all light intensities as well as the largest xanthophyll pool,

providing constitutive quenching. It is generally accepted that a higher amount of zeaxanthin, most efficiently bound to oligomeric LHCII (Johnson *et al.*, 2007), prevents singlet oxygen formation in the antenna (Demmig-Adams and Adams, 2002; Adams *et al.*, 2004). We suggest that under SD/HT conditions, having a very low DEPS, singlet oxygen produced via triplet chlorophyll could be quenched by a higher amount of β-carotene (Fig. 18C), an excellent quencher of singlet oxygen (Cantrell *et al.*, 2003; Krieger-Liszkay, 2005; Telfer, 2005).

Changes in 77 K fluorescence emission characteristics of the long wavelength peak were observed and a shift of the emission maximum from 731 nm (LD/HT and SD/HT) to 723 nm (SD/LT) and 713 nm (LD/LT) was detected. Although it has been shown that this could be due to changes in the LHCI composition (Bossmann *et al.*, 1997; Zhang *et al.*, 1997), this is most likely not the case here, since we previously found no significant changes in the relative amount of LHCI components (Busch *et al.*, 2007). We attribute the blue-shift to a disconnection of LHCI from PSI as it has been previously observed in iron deficient *Chlamydomonas* (Moseley *et al.*, 2002), in the red algae *Rhodella violacea* (Desquilbet *et al.*, 2003) and in lincomycin treated maize (*Zea mays*; Gáspár *et al.*, 2006).

Redox regulation and electron transport under SD/HT conditions

Plants employ a multi step regulation to balance the electron flow with the required rate of ATP and NADPH production under changing environmental conditions. These include D1 turnover, state transitions, NPQ, xanthophyll cycle, chlororespiration, Mehler reaction, CEF flow and reactive oxygen species production (Scheibe *et al.*, 2005). It has been shown that endogenous systems that measure daylength interact with redox regulation (Becker *et al.*, 2006; Queval *et al.*, 2007). We show for the first time that increased temperature under a short, autumn photoperiod (SD/HT) results in higher excitation pressure than in summer control plants (LD/HT) (Fig. 20C), i.e. an over-reduction of the PQ pool. This appears to be due to limitations in intersystem electron transport and CO_2 assimilation as a consequence of alterations in the structure and composition of the photosynthetic apparatus. PTOX should oxidize an over-reduced PQ pool, which should be associated with lower excitation pressure (Rosso *et al.*, 2006). We observed

a 3-fold increase in the accumulation of PTOX under SD/HT and low temperature conditions as compared to the summer control plants (LD/HT) (Fig. 17G). Although the intersystem electron pool size (e^-/P700) was 44% lower in plants exposed to low temperature (Fig. 21; LD/LT, SD/LT), e^-/P700 was similar in SD/HT compared to LD/HT plants (Fig. 21). Furthermore, excitation pressure, measured as $1-q_P$, was 1.4-fold higher in SD/HT plants and about 3-fold higher in LD/LT and SD/LT plants than summer control plants (LD/HT) (Fig. 20C). Although PTOX accumulation is stimulated by stress (Fig. 17G), the accumulation of PTOX does not alleviate excitation pressure in *P. banksiana*. Thus, PTOX does not appear to act as a safety valve under conditions of environmental stress in *P. banksiana*, as suggested by Rosso *et al.* (2006) for Arabidopsis. Alternatively, the higher levels of PTOX may support a higher production of carotenoids by phytoene desaturase in *P. banksiana*. Shahbazi *et al.* (2007) reported that PTOX is involved in both redox regulation as well as in carotenoid desaturation in support of the suggestion of Peltier and Cournac (2002). Clearly, the functional role of PTOX remains an enigma.

Conclusions

In summary, we have shown that the inhibition of CO_2 assimilation in *Pinus banksiana* associated with exposure to elevated temperatures during a short, autumn photoperiod is a consequence of an inhibition of photosynthetic electron transport associated with a decreased PSII/PSI stoichiometry coupled with decreased levels of Rubisco. Furthermore, the results presented are consistent with the model for zeaxanthin-independent antenna quenching presented previously (Busch *et al.*, 2007). A major component of photoprotection under short, autumn photoperiod appears to be due to antenna quenching through zeaxanthin-independent LHCII aggregation to balance light absorption with a decreased capacity for energy utilization through CO_2 assimilation. In *Pinus banksiana*, elevated temperatures and a short, autumn photoperiod interact to affect the structure and function of the photosynthetic apparatus which leads to an inhibition of CO_2 assimilation. The results from our experiments using seedlings grown in controlled environments with a relatively large difference in air temperature do not allow any quantitative prediction of how entire boreal forest stands might be affected by

the projected increase of autumn air temperature of 4-7 °C by 2100 (ACIA, 2005; IPCC, 2007). However, the experiment clearly demonstrates the significance of warm autumn temperatures on the cold hardening process in conifers which warrant further investigation, e.g. under manipulated field conditions. A net CO_2 loss in response to autumn warming was recently observed in the field in northern ecosystems, and the underlying mechanisms and processes have yet to be explained (Piao *et al.*, 2008). Models which predict effects of climate change on conifers of the boreal forests mostly fail to take into account the interactive effects of increased temperatures and short photoperiod (Heimann and Reichstein, 2008). We suggest that the understanding of such an interaction is of critical importance for future modelling of the response of boreal forests of the northern hemisphere to global warming.

Materials and Methods

Plant material and growth conditions

One year old rooted Jack pine (*Pinus banksiana* Lamb.) seedlings were obtained from a local nursery (Somerville Seedlings, Everett, ON, Canada) and planted in a mixture of ProMix (Premier Horticulture Inc., Quakertown, PA, USA) and low nutrient mineral sand (1:2, v/v). The plants were kept outside underneath a light shelter for two years. In the third year, the plants were transferred to controlled environments in late summer 2006 (Conviron growth chambers, Winnipeg, MB, Canada). Eight three year old plants per treatment were exposed for four weeks to either 22 °C/18 °C (day/night) with a photoperiod of 16 h (LD/HT, representing summer), 22 °C/18 °C with 8 h photoperiod (SD/HT, warm autumn condition), 7 °C/5 °C with 16 h photoperiod (LD/LT, cold summer condition) and 7 °C/5 °C with 8 h photoperiod (SD/LT, representing late autumn). The photosynthetic photon flux density was set to 500 μmol photons m^{-2} s^{-1} for all four treatments. Although light intensity and quality may not accurately reflect field conditions, a factorial design with temperature and photoperiod as the only two variables allows for a qualitative analysis of the effect of increased autumn air temperature on evergreen conifers.

Protein extraction, SDS-PAGE and immunoblotting

For protein extraction, needles were ground to a fine powder in liquid nitrogen. The proteins were extracted as described in detail earlier (Busch *et al.*, 2007). To determine the total concentration of extracted protein a test after Lowry *et al.* (1951) was performed, using the RC DC protein assay kit from Biorad (Hercules, CA, USA). Seven μg of protein per lane were loaded and separated electrophoretically at 200 V for 30 min on 10 % (w/v) Bis-Tris gels (Nupage, Invitrogen, Carlsbad, CA, USA) using the XCell Midi gel system and a MES/SDS buffer system (Invitrogen, Carlsbad, CA, USA). The proteins were then transferred to a nitrocellulose membrane (0.2 μm pore size, Biorad, Hercules, CA, USA) and probed with antibodies against Lhcb1, PsbA, Cyt *f*, plastocyanin, RbcL (Agrisera, Vännäs, Sweden) as well as against PSI and PTOX. Goat anti-rabbit and rabbit anti-chicken IgG conjugated with horseradish peroxidase (Sigma-Aldrich Inc., St. Louis, MO, USA) were used as secondary antibodies to allow for chemiluminescent detection (ECL detection kit, GE Healthcare, Buckinghamshire, UK) of the proteins bound to the membrane. The membranes treated this way were exposed to x-ray film (Super RX, Fujifilm, Tokyo, Japan). The optical density of each band on the film was quantified using the Scion software package (Scion Corp., Frederick, MD, USA).

Photosynthetic pigments

Needles of plants of all treatments were detached around noon when plants had been already exposed to growth light for 4 h. They were then put on trays with their bottom covered with wet filter paper and exposed to 100, 200, 400, 800 and 1500 μmol photons $m^{-2}\,s^{-1}$. After 2 h of exposure to the respective light intensities the samples were frozen in liquid nitrogen and stored at -80 °C until further analysis. In addition, one set of samples was taken in the morning before the light was turned on to get dark adapted samples. Needles were ground to a fine powder in liquid nitrogen and pigments were extracted for 2 hours in the dark on ice in 100 % acetone buffered with $NaHCO_3$. The pigment extracts were separated by high-performance liquid chromatography (HPLC) as described by (Busch *et al.*, 2007). The deepoxidation state was calculated as DEPS = (0.5 A + Z)/(V + A + Z). V, violaxanthin; A, antheraxanthin; Z, zeaxanthin.

Chl Fluorescence measurements

Chlorophyll a fluorescence was measured with a PAM 2100 chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany). F_0 (initial minimal fluorescence) and F_m (maximum fluorescence) were determined in the morning at the end of the dark period. F_0' (minimal fluorescence immediately after illumination), F_m' (maximum fluorescence under actinic light) and F_t (transient fluorescence) were recorded after steady state fluorescence was achieved, usually within a 3 min illumination period. Optimum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$ and the effective quantum yield of PSII in the light as $F_v'/F_m' = (F_m' - F_t)/F_m'$ (Genty *et al.*, 1989). The fraction of PSII RCs in a closed state was estimated as $1-q_P = 1-(F_m' - F_t)/(F_m' - F_0')$ (Schreiber and Bilger, 1987). Non-photochemical quenching was calculated as $NPQ = F_m/F_m' - 1$ (Bilger and Björkman, 1990) and antenna quenching as $q_O = 1 - F_0'/F_0$ according to Rees *et al.* (1990).

To assess the relaxation of F_0 quenching the plants were dark adapted for 20 min with a dark leaf clip and subsequently exposed to 650 μmol photons $m^{-2}\,s^{-1}$ for 4.5 min. The postillumination relaxation of Chl fluorescence was followed at the F_0' level after switching off the actinic light with and without simultaneously applying FR light.

P700 Measurements

The redox state of P700 was determined *in vivo* using a PAM-101 modulated chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany) equipped with an ED-P700DW detector following the procedure of Schreiber *et al.* (1988) as described in detail by Ivanov *et al.* (1998). Far red light (λ_{max} = 715 nm, 10 Wm^{-2}, Schott filter RG 715) was provided by the 101-FR light source. MT (multiple turnover, 50 ms) and ST (single turnover, half-peak width 14 μs) saturating flashes were applied with the Walz XMT-103 power unit and the Walz XST-103 control unit. The redox state of P700 was measured on detached needles as the absorbance change around 820 nm ($\Delta A_{820}/A_{820}$) at growth temperature (22 °C or 7 °C). The transient reduction of $P700^+$ signal after application of single- and multiple-turnover flashes of white saturating light was used to estimate the intersystem electron pool size (Asada *et al.*, 1993; Ivanov *et al.*, 1998).

Low temperature (77 K) fluorescence measurements

Low temperature (77 K) chlorophyll fluorescence emission spectra were collected using a PTI QM-7/2006 spectrofluorometer (Photon Technology International, South Brunswick, NJ, USA) equipped with a double monochromator, R928P red-sensitive photomultiplier tube (Hamamatsu Photonics, Shizuoka-ken, Japan) and a liquid nitrogen device. Thylakoid membranes suspended in a buffer containing 35 mM Tricine (pH 7.8), 0.3 M sorbitol, 7 mM NaCl, 3.5 mM $MgCl_2$ were dark adapted for 30 min and frozen in the presence of 30 % glycerol before the measurements. The Chl concentration was 5 $\mu g\,ml^{-1}$. Corrected fluorescence emission spectra were excited at 436 nm and recorded from 650 nm to 800 nm using a slit width of 2.5 nm for both excitation and emission. All fluorescence spectra were additionally corrected by subtracting the medium blank.

Statistics

The effects of daylength and temperature on photosynthetic properties were statistically analyzed by two-way ANOVA at $P < 0.05$ using SPSS Version 14.0 (Chicago, Il, USA). All significant differences mentioned in the text and the figures refer to the two-way ANOVA results.

Acknowledgment

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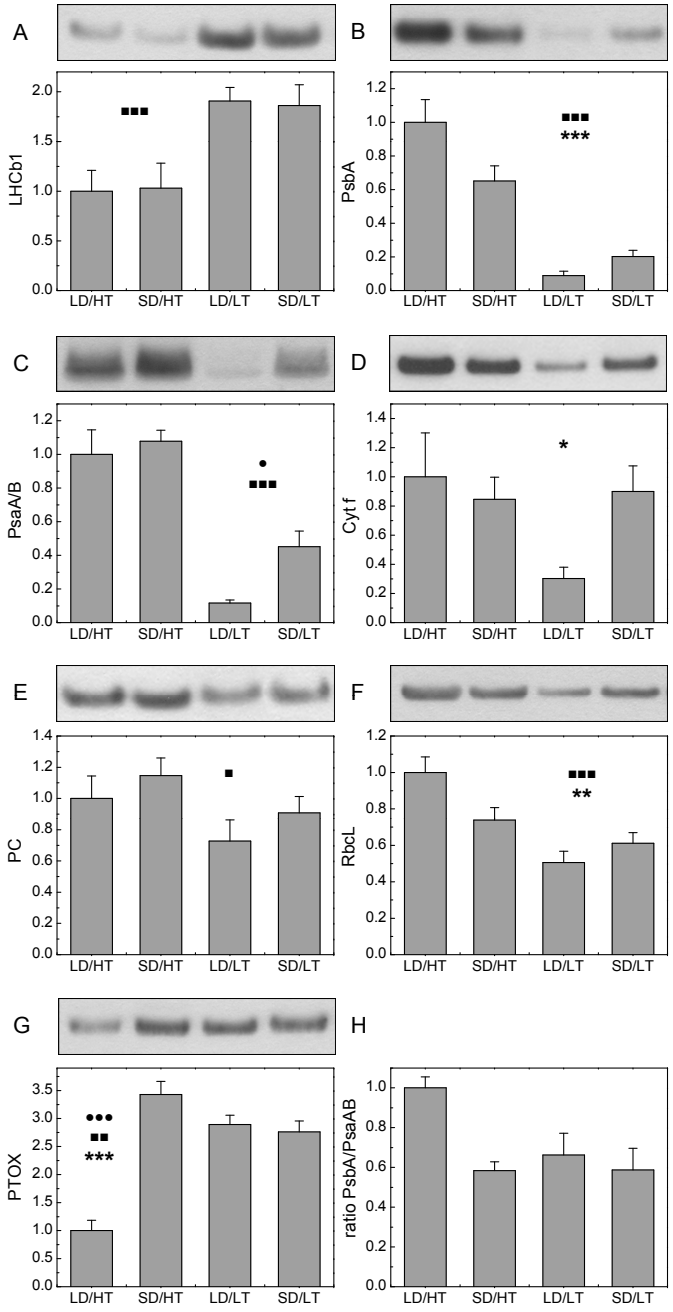

Figure 17: *The effect of daylength and temperature on the expression levels of key proteins of the photosynthetic electron transport chain in needles of* P. banksiana. *The average optical density of the LD/HT treatment was arbitrarily scaled to 1. Typical bands from the original western blots, loaded on an equal protein basis, are shown next to the values. Each value represents the average of n = 8 ± SE biological replicates. Two-way ANOVA analysis indicates statistically significant differences due to daylength, temperature or an interactive effect of both factors. ●, ■, and ⋆ indicate significant differences due to daylength, temperature and their interactive effect respectively. One symbol: P < 0.05; two symbols: P < 0.01; three symbols: P < 0.001.*

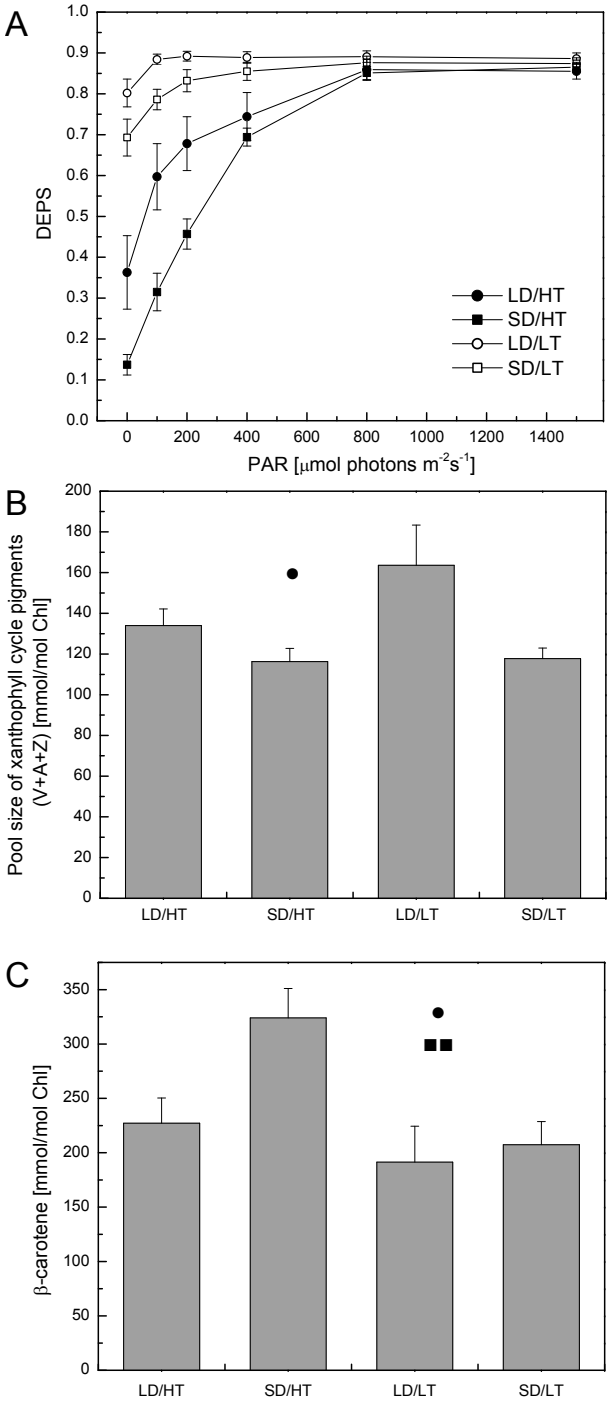

Figure 18: *The effect of daylength and temperature on the composition of photosynthetic pigments in needles of* P. banksiana. *(A) deepoxidation status of the xanthophyll cycle pigments (DEPS) in response to PAR, calculated as (0.5 A + Z)/(V + A + Z). Needles were exposed to the corresponding light intensity for 2 h before harvesting; (B) total pool size of xanthophyll cycle pigments (V + A + Z) per total chlorophyll; (C) amount of β-carotene per total chlorophyll; Each data point represents the average of n = 8 ± SE biological replicates. ●, ■, and ⋆ indicate significant differences due to daylength, temperature and their interactive effect respectively. One symbol: P < 0.05; two symbols: P < 0.01.*

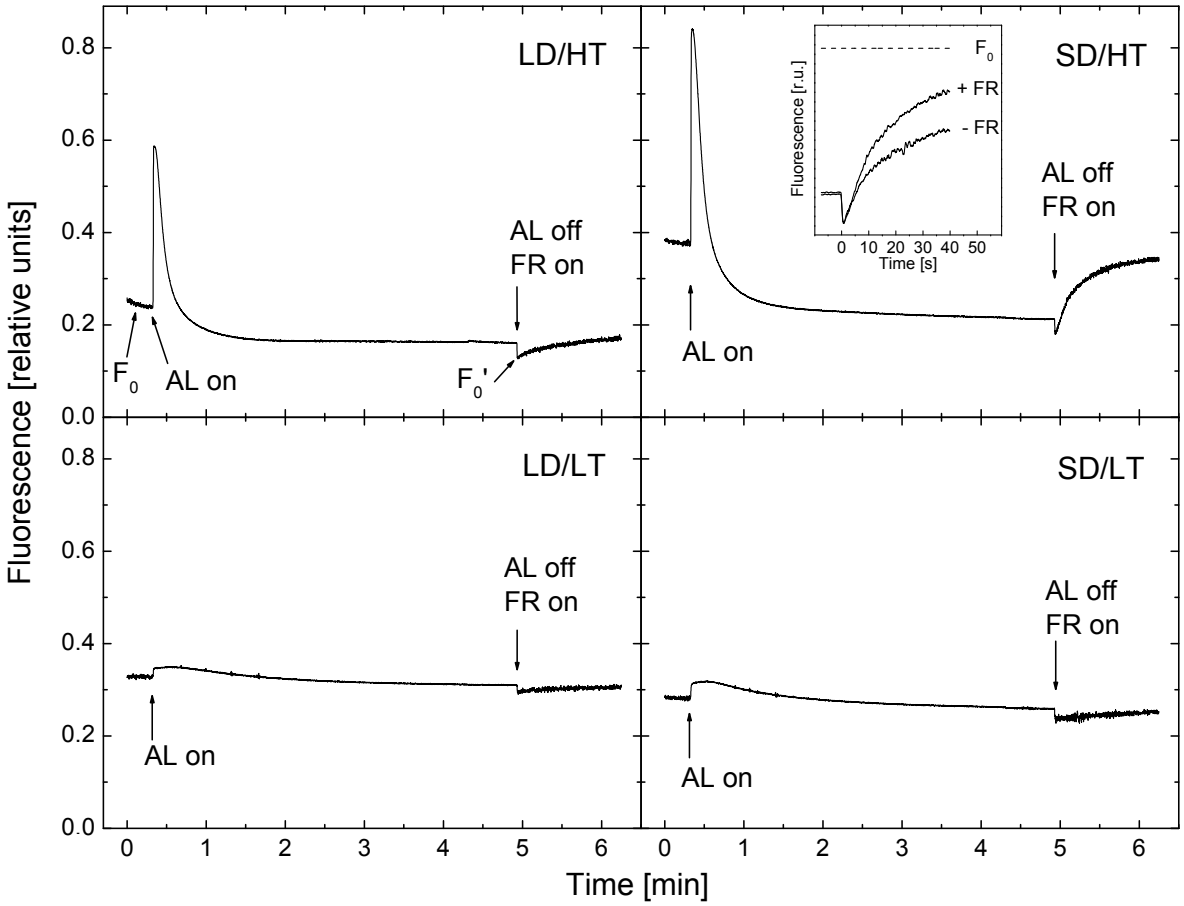

Figure 19: *Fluorescence transients of* P. banksiana *needles grown under LD/HT, SD/HT, LD/LT and SD/LT conditions. Dark adapted needles were exposed to actinic light (AL) of 650* μmol photons $m^{-2} s^{-1}$ *for 4.5 min before the light was turned off and far red light (FR) was turned on. Traces are averages of five independent biological replicates. The insert shows the rate of fluorescence recovery during the first 40 s after turning off AL in SD/HT with and without applying FR light.* F_0*, basic fluorescence of the dark adapted sample. Please note that the fluorescence transients shown in this figure do not result from saturating flash induced fluorescence kinetics as e.g. in Fig. 20 (See material and methods for further details).*

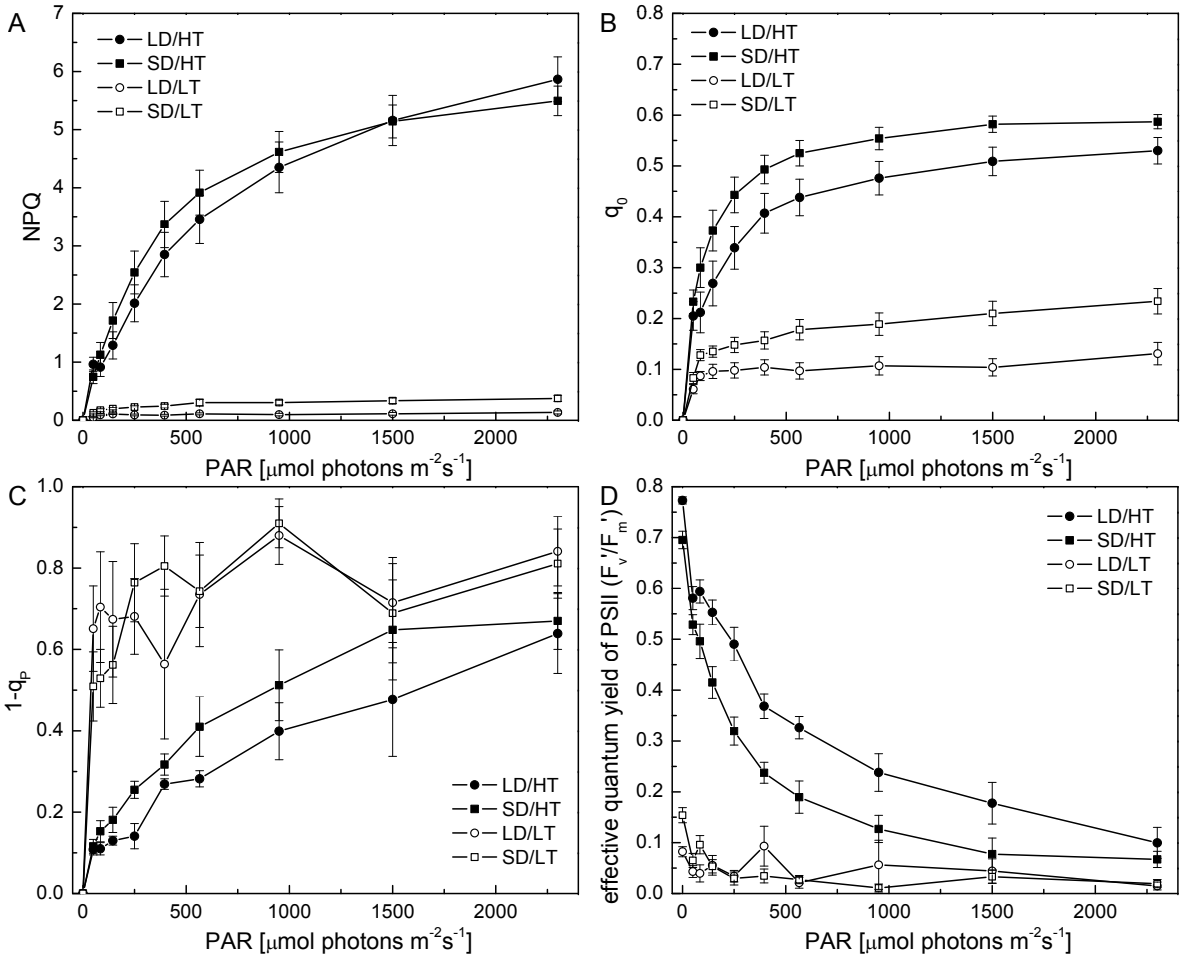

Figure 20: *The effect of daylength and temperature on Chl fluorescence parameters in response to PAR. (A) Light saturation curve for non-photochemical quenching (NPQ); (B) light saturation curve for antenna quenching (q_O); (C) Estimated fraction of closed PSII reaction centers ($1-q_P$); (D) Effective quantum yield of PSII (F_v'/F_m'). All measurements were performed at growth temperature (22°C for LD/HT and SD/HT and 7°C for LD/LT and SD/LT) at the end of the night on dark adapted seedlings. Each data point represents the average of n = 6 to 8* ± SE *biological replicates.*

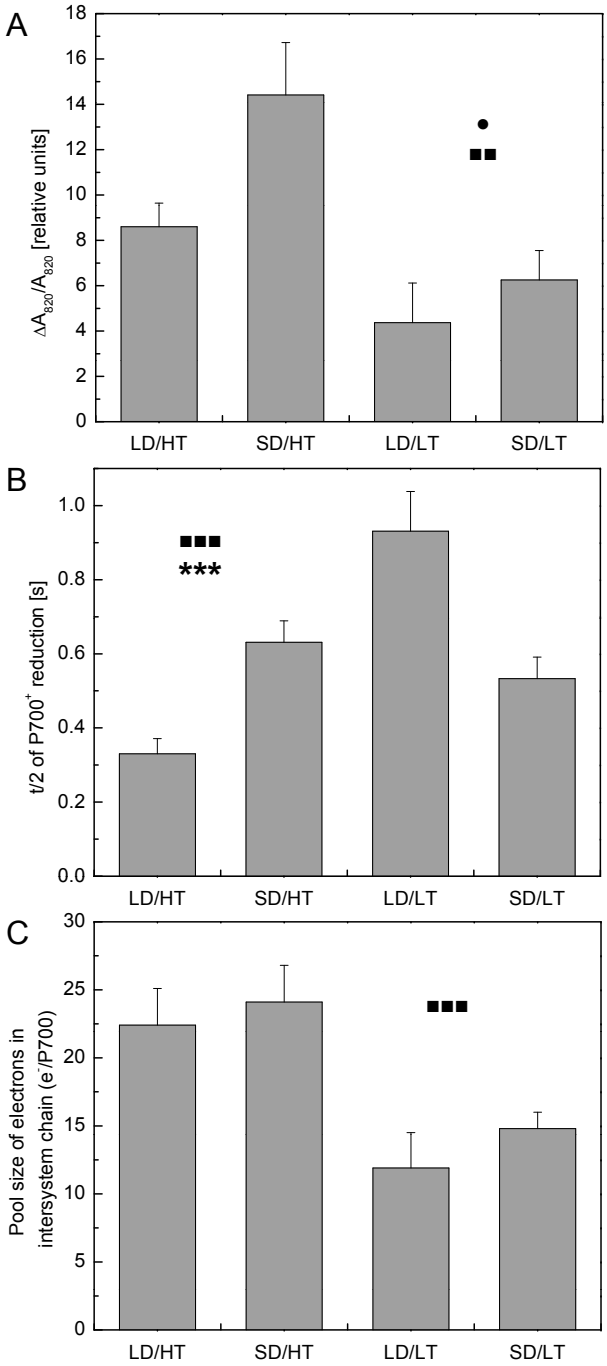

Figure 21: *The effect of daylength and temperature on* in vivo *P700 parameters in* P. banksiana *needles grown under different daylength and temperature regimes. After a steady state level of* $P700^+$ *was achieved under FR illumination, ST and MT pulses were applied. (A) Steady-state oxidation of P700 (*$\Delta A_{820}/A_{820}$*); (B) Reduction kinetics of* $P700^+$ *after the FR light was turned off (*$t_{1/2}$*); (C) Effective intersystem electron pool size (*e^-*/P700). All measurements were performed at the growth temperature. Each data point represents the average of n = 6 to 8* ± SE *biological replicates.* ●, ■, *and* ⋆ *indicate significant differences due to daylength, temperature and their interactive effect respectively. One symbol: P < 0.05; two symbols: P < 0.01; three symbols: P < 0.001.*

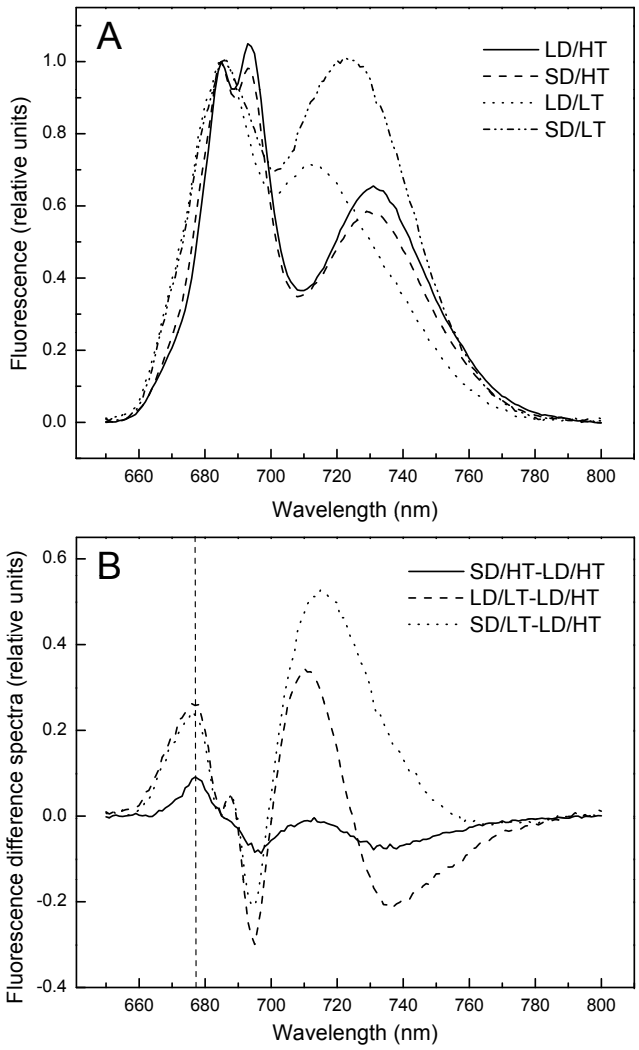

Figure 22: *The effect of daylength and temperature on low temperature (77 K) fluorescence emission properties in* P. banksiana *needles grown under different daylength and temperature regimes. (A) Fluorescence emission spectra (excited at 436*nm*) of the four different treatments. The spectra were normalized to the peak at 685*nm*. Each trace represents the average of n = 8 independent biological replicates. (B) Corresponding difference spectra SD/HT - LD/HT, LD/LT - LD/HT, SD/LT - LD/HT. The Chl concentration of all samples was 5 µg Chl* ml^{-1}.

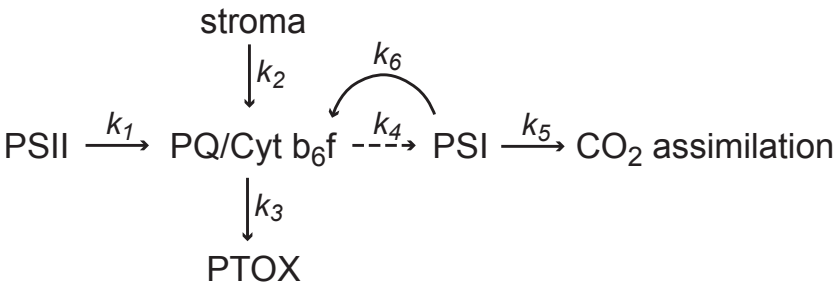

Figure 23: *Possible points of regulation for photosynthetic electron transport.* k_1 *to* k_6 *indicate rate constants for the flux of electrons.* k_4 *is highlighted by a dashed line, as this is the probable site of limitation in photosynthetic electron transport. See text for further details.*

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IV

IV

Temperature and light differentially affect light use efficiency when estimated either by chlorophyll a fluorescence or leaf spectral reflectance during the photosynthetic recovery of winter acclimated Jack pine

Submitted to Functional Plant Biology

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Abstract

Leaf reflectance spectral measurements are an emerging non-invasive technique which can be used to derive the photosynthetic reflectance index (PRI), allowing to assess the physiological state of plants from leaf to ecosystem level. Changes in PRI are associated with changes in the xanthophyll cycle activity and provide an estimate of changes in photosynthetic light use efficiency (LUE) during the growing season. However, we hypothesize that the correlation between PRI and LUE might be poor when the xanthophyll cycle is arrested. This is particularly important in boreal evergreens during the winter to spring transition, since they retain their leaves throughout the winter. To test our hypothesis, we studied the recovery of winter acclimated Jack pine (*Pinus banksiana* Lamb) seedlings that were exposed to different simulated spring recovery treatments in controlled environments. Three different growth temperatures and three light intensities were used to dissect the effect of these two factors on chlorophyll fluo-

rescence, pigment composition and leaf reflectance. The electron transport rate (ETR) as a measure of photosynthetic recovery showed a clear response to temperature alone, whereas PRI was only affected by the light intensity. In contrast, zeaxanthin levels during the recovery period were both, temperature and light dependent. As a result, during the experimental winter to spring transition, PRI could not explain the variation in ETR. Our data indicate that an improved understanding of the different energy quenching mechanisms is critical to accurately interpret the PRI signal under environmental conditions where the predominant mode of excess energy dissipation does not involve a dynamic xanthophyll cycle.

Introduction

Photosynthesis is strongly influenced by environmental conditions and varies over the course of a day as well as between seasons (Ensminger *et al.*, 2001, 2004). It plays a major role in the global carbon cycle while at the same time it is very sensitive to climate variability (Barr *et al.*, 2004; Amiro *et al.*, 2006). Thus, models that attempt to predict the uptake of CO_2 and the production of biomass rely on an accurate understanding of atmosphere-biosphere interactions and the feedback control of environmental or seasonal factors on photosynthetic capacity. The boreal forest, representing one of the largest biomes in the world, plays an important role in the global carbon cycle (Hall *et al.*, 2004). By changing precipitation patterns as well as increasing the length of the growing season due to changes in air and soil temperatures, global climatic change will affect the spatial and temporal characteristics of the carbon cycle of boreal forests. To assess accurately carbon cycling, data on the photosynthetic capacity over large spatial scales are necessary. However, monitoring the spatial and temporal variation of photosynthetic capacity is time consuming and requires enormous resources. A non-invasive technique to measure plant performance is chlorophyll fluorescence, which has established itself as a proxy for functional properties and changes in the photosynthetic apparatus (Demmig-Adams and Adams, 1996; Verhoeven *et al.*, 1996). Despite recent attempts to establish methods to remotely assess chlorophyll fluorescence (Ananyev *et al.*, 2005; Soukupová *et al.*, 2008), such techniques still do not allow proper estimates of chlorophyll fluorescence parameters on larger scales, e.g. stand or ecosystem scale.

Leaf reflectance measurements have recently emerged as another non-invasive technique to evaluate the physiological state of plants. A reflectance-based photosynthetic parameter, the photochemical reflectance index (PRI), was developed to provide an indication of photosynthetic light use efficiency (LUE), i.e. the amount of CO_2 assimilation per mol of photosynthetically active radiation absorbed (Gamon *et al.*, 1990, 1992). PRI is based on the changes in reflectance (R) at 531 nm relative to a reference band at around 570 nm. The change in reflectance at 531 nm is a measure of xanthophyll cycle activity through the conversion of the light harvesting pigment, violaxanthin, to the deepoxidized, energy quenching pigments, antheraxanthin and zeaxanthin (Gamon *et al.*, 1992). This conversion is indicative of non-photochemical quenching (NPQ), a photoprotective mechanism, which dissipates excess light energy as heat (Demmig-Adams and Adams, 1996). Several studies have shown that changes in the antheraxanthin and zeaxanthin levels closely match NPQ as a measure of the rate constant for heat dissipation (Demmig-Adams and Adams, 1996; Niyogi *et al.*, 1998). It has also been shown, both at the leaf and ecosystem level, that PRI correlates with zeaxanthin-dependent NPQ and LUE, (Gamon *et al.*, 1992; Peñuelas *et al.*, 1995, 1997; Stylinski *et al.*, 2002; Trotter *et al.*, 2002). As a consequence, PRI has been used to estimate LUE, which is, in turn, used to estimate photosynthetic activity on an ecosystem scale.

During winter, photosynthesis is downregulated in many perennial plant species, in connection with a low PSII efficiency and the accumulation of large amounts of zeaxanthin as compared to the summer (Verhoeven *et al.*, 1996; Adams *et al.*, 2001; Ensminger *et al.*, 2004, 2006). For short-term exposure to excess energy, the flexible, zeaxanthin-dependent mechanism for NPQ is important to balance the energy flow. Evergreens of the boreal forest, such as *Pinus banksiana*, retain their leaves throughout the winter and resume photosynthesis once conditions become favourable in the spring. In winter, when the intrinsic photosynthetic capacity is downregulated and most of the light energy cannot be utilized in photosynthetic carbon fixation, the rapidly reversible dissipation of excess energy through the xanthophyll cycle is diminished and replaced by a more sustained NPQ, which relies on PSII core rearrangements and a decrease in the conversion of zeaxanthin to violaxanthin (Demmig-Adams and Adams, 2006). This results in a sustained photoinhibition as indicated by suppression of predawn F_v/F_m val-

ues and is a major component of the protection of the photosynthetic apparatus (Hurry *et al.*, 1992; Adams and Demmig-Adams, 1994; Repo *et al.*, 2005; Ensminger *et al.*, 2008). However, in addition to these zeaxanthin-dependent quenching mechanisms, overwintering evergreens also dissipate excess energy through zeaxanthin-independent aggregation of LHCII complexes (Horton *et al.*, 1991; Gilmore and Ball, 2000; Busch *et al.*, 2007) as well as through reaction center quenching (Ivanov *et al.*, 2002). Since PRI reflects changes in the zeaxanthin content, these quenching mechanisms, which affect LUE, are not reflected in the spectral reflectance that is used to derive the PRI signal.

The relationship between PRI and chlorophyll fluorescence parameters is quite well documented for a range of plant species and plant functional types during the growing season, when the xanthophyll cycle operates dynamically with the formation of zeaxanthin during stress and its rapid re-conversion via antheraxanthin to violaxanthin (Gamon *et al.*, 1990, 2001). In contrast, the relationship between photosynthesis, PSII activity and PRI has not yet been assessed during the transition from winter dormancy, when the xanthophyll cycle is still arrested in a state primed for the sustained dissipation of excess energy, to the initiation of growth and photosynthesis in the spring. This transition is accompanied by a complete change in function of the xanthophyll cycle. Since in winter, sustained zeaxanthin-dependent and -independent quenching mechanisms predominate rather than flexible photoprotection, PRI may over-estimate LUE and hence over-estimate photosynthetic potential. Thus, the goal of this study was to assess the relationship between PRI, PSII photochemistry and NPQ as estimated by chlorophyll fluorescence during the recovery of photosynthesis after winter-induced downregulation of photosynthesis in *Pinus banksiana*, one of the main conifer species in western boreal forests.

Materials and Methods

Plant material and growth conditions

One year old, rooted Jack pine (*Pinus banksiana* Lamb.) seedlings were obtained from a local nursery (Somerville Seedlings, Everett, ON, Canada) and planted in a mixture of ProMix (Premier Horticulture Inc., Quakertown, PA, USA) and low nutrient mineral

sand (1:2, v/v). The plants were kept outside in an experimental field at the University of Western Ontario (London, ON, Canada) from October 2004 until they were transferred to controlled environments (Conviron growth chambers, Winnipeg, MB, Canada) immediately prior to the start of the experiment on 18 March 2005. At the time of transfer, the outside temperature was -7 °C and the plants were partly covered with snow. To simulate possible spring recovery conditions, seedlings were randomly assigned to one of three different temperature regimes (5 °C, 10 °C and 15 °C) as well as one of three different light intensities (50, 250 and 500 μmol photons $m^{-2} s^{-1}$), all with a photoperiod of 12 h. The recovery of photosynthesis was followed by taking chlorophyll fluorescence measurements before the transfer as well as after 6 h, 12 h, 24 h, 2 days, 4 days, 8 days and 12 days after the transfer to simulated spring recovery conditions. Reflectance was measured and pigment samples were taken before the transfer as well as after 6 h and 12 days after the transfer to simulated spring recovery conditions.

Meteorological Data

Daily average air temperature and snow cover over the course of the cold acclimation period were obtained from the nearby Environment Canada weather station at the London airport, London, Canada.

Chl Fluorescence measurements

For each sampling time point, chlorophyll fluorescence was measured with a PAM 2000 chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany). F_0 (initial minimal fluorescence) and F_m (maximal fluorescence) were determined after a 20 min dark period. F_0' (minimal fluorescence immediately after illumination), F_m' (maximal fluorescence under actinic light) and F_t (transient fluorescence) were obtained when steady state of the fluorescence trace was achieved, which usually occurred within 4 min after the start of illumination with 500 μmol photons $m^{-2} s^{-1}$. Optimum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$ and the quantum yield of PSII in the light as $F_v'/F_m' = (F_m' - F_t)/F_m'$ (Genty *et al.*, 1989). The rate of electron transport was calculated as ETR = PAR $*$ F_v'/F_m'. The excitation pressure was estimated as $1-q_P$

$= 1-(F_m' - F_t)/(F_m' - F_0')$ (Schreiber and Bilger, 1987). Non-photochemical quenching was calculated as NPQ = $(F_m - F_m')/F_m'$ (Bilger and Björkman, 1990).

Photosynthetic pigments

After an exposure to growth light for 4 h needle samples were taken along with the fluorescence measurements. The needle samples were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Needles were then ground to a fine powder in liquid nitrogen and the pigments were subsequently extracted for 2 hours in the dark on ice in 100 % acetone buffered with $NaHCO_3$. Extracts were separated by high-performance liquid chromatography (HPLC) as described by Busch *et al.* (2007). The deepoxidation state (DEPS) was calculated as DEPS = (0.5 A + Z)/(V + A + Z). V, violaxanthin; A, antheraxanthin; Z, zeaxanthin.

Spectral measuremtents of needle reflectance

Leaf reflectance was determined with a portable spectroradiometer (Unispec UNI003 Spectral Analysis System, PP Systems, Haverhill, MA, USA) fitted with a bifurcated fiber optic (UNI400) and a leaf clip (UNI500). Reflectance measurements were made using the built-in halogen light source. Leaf reflectance was derived from leaf radiance divided by radiance of a 99 % reflective polytetrafluoroethylene (PTFE) calibration disk (UNI420) that had been calibrated relative to a diffuse reflective surface made of 99 % reflective PTFE (Spectralon, Labsphere Inc., North Sutton, NH). Leaf radiance measurements were preceded with dark current noise measurements and a measurement of the calibration disk. Measurements were recorded as the average of 10 scans. Spectral resolution of the spectroradiometer was 10 nm across the useable range between 400 and 1000 nm. The data were interpolated to 1 nm bandwidths. PRI was then calculated from the leaf reflectance data according to Gamon *et al.* (1997) as:

$$PRI = (R_{531} - R_{570})/(R_{531} + R_{570})$$

where R refers to reflectance and the subscript refers to the wavelength in nanometers.

Statistics

The effects of temperature and light intensity on reflectance and photosynthetic properties were statistically analyzed by two-way ANOVA at $P < 0.05$ using SPSS Version 14.0 (Chicago, Il, USA).

Results

Outdoor growth conditions of the pine seedlings between October 2004 and March 2005 prior to transfer to experimental conditions are given in Fig. 24. Throughout most of the winter the seedlings experienced freezing temperatures combined with snow covering the plants at least partially until the time of transfer on 18 March 2005. During this period, F_v/F_m decreased from 0.727 ± 0.017 in early October to a cold acclimated value of 0.227 ± 0.032 at the time of transfer (Fig. 25A, Time 0). Once the cold acclimated plants were transferred to controlled environments, the recovery of photosynthesis was followed over a period of 12 days by measuring chlorophyll fluorescence. During the 12 day recovery from winter stress, the rate and extent of recovery of F_v/F_m was clearly temperature dependent, whereas light intensity appeared to exert a minimal effect on the recovery of F_v/F_m irrespective of temperature (Fig. 25A). After 12 days, the lowest values of F_v/F_m were attained at 5 °C, intermediate values at 10 °C and the highest values at 15 °C (Fig. 25E). In the cold acclimated state the plants exhibited a low ETR. As with F_v/F_m, the recovery of ETR was largely temperature dependent (Fig. 25B). Exposure of seedlings to 15 °C resulted in ETR values being roughly three times of the values that were observed in plants exposed to 5 °C, with plants exposed to 10 °C being intermediate (Fig. 25F). Similar to F_v/F_m and ETR, NPQ in cold acclimated seedlings was relatively low and the recovery of NPQ was largely temperature dependent (Fig. 25C). However, in contrast to ETR, after 12 days NPQ values indicate an effect of both, light and temperature (Fig. 25G). $1-q_P$ remained at the level of the cold acclimated plants, independent of the light intensity, during the recovery at 5 °C. Higher temperatures resulted in a 27 to 50 % decrease of excitation pressure (Fig. 25D). After 12 days of recovery $1-q_P$ was clearly temperature dependent, whereas the light intensity did not appear to have a significant effect (Fig. 25H).

Within 12 days of recovery, major changes in pigment content and composition occurred. As with the recovery of F_v/F_m, changes in the total xanthophyll cycle pigment pool size were strongly dependent on temperature, but not on light intensity (Fig. 26A). Compared to the cold acclimated seedlings, no change in the xanthophyll cycle pool size was observed at 10 °C. However, a 19 to 23 % increase at 5 °C was detected and a 6 to 21 % decrease was observed during the recovery at 15 °C, irrespective of the light intensity (Fig. 26A). Zeaxanthin was the most abundant xanthophyll cycle pigment in cold acclimated plants, accounting for 53 % of the total xanthophyll cycle pigments (Fig. 26A,B). Upon transfer of the plants from the winter to the recovery conditions, zeaxanthin levels dropped to 8 to 49 % of the cold acclimated level. After 12 days the highest level of zeaxanthin was observed during recovery at 5 °C at all light intensities (16 to 49 % of cold acclimated value) and decreased to a larger extent during recovery at the warmer temperatures (8 to 16 % of cold acclimated value) (Fig. 26B). By far the highest amount of zeaxanthin was observed during recovery at 5 °C/500 μmol photons $m^{-2} s^{-1}$. The change in the deepoxidation state of the xanthophyll cycle pigments during recovery paralleled the zeaxanthin pattern (Fig. 26C). Fig. 26D depicts the amounts of Chl a + b per fresh weight. At 5 °C the amount of Chl a + b did not change upon exposure to recovery conditions, when compared to the cold acclimated winter state. However, at higher temperatures, we observed about a 48 % increase in the Chl content, making the accumulation of Chl also primarily dependent on temperature (Fig. 26D).

The cold acclimated plants showed the lowest PRI values and the PRI values increased during the recovery period, with lower light intensities corresponding to higher PRI values (Fig. 27). The only exception from this general trend was observed in the 15 °C treatments, where the same amount of PRI was measured irrespective of the growth light intensity. In contrast to the recovery of F_v/F_m, ETR, NPQ and excitation pressure, which were largely temperature dependent, the recovery of PRI was solely light dependent (Fig. 27).

Fig. 28 demonstrates the relationship between photosynthetic electron transport (ETR), and the reflectance parameter PRI for three different time points – cold acclimated winter plants as well as 6 h and 12 days after the transfer to experimental spring conditions. Considering all measurements throughout the whole recovery period, PRI only weakly correlated with ETR (solid line, $R^2_{all} = 0.324$). The highest correlation was found under cold acclimated conditions (blue dashed line, $R^2_{CA} = 0.543$), where PRI occurred virtually independently of ETR. Six hours after transfer from winter to recovery conditions, ETR had already started to recover, while the PRI values remained comparable to the cold acclimated values. Twelve days after the transfer, both, ETR and PRI, increased. However, the correlation between these two parameters was extremely weak (6 h: yellow dashed line, $R^2_{6h} = 0.086$ and 12 d: red dashed line, $R^2_{12d} = 0.046$) (Fig. 28).

Discussion

In many plant species grown under a variety of environmental conditions, PRI has been shown to be linearly correlated with the effective quantum yield of PSII (Peñuelas *et al.*, 1995). It is not always feasible to directly measure the quantum yield, since the remote assessment of photosynthesis via fluorescence parameters requires an active measuring light and is therefore limited to smaller scale applications. PRI measurements using leaf reflectance spectral data represent a passive system, which is independent of an active measuring light. This opens the opportunity of assessing plant productivity on much larger spatial scales through studies from ground- (Filella *et al.*, 2004), tower- (Gamon *et al.*, 1992) or air-borne (Nichol *et al.*, 2000) to even whole ecosystems by using remote sensing platforms on satellites (Drolet *et al.*, 2005). However, in the following discussion, we dissect the impact of temperature and light intensity on the various photosynthetic parameters of the pine needles and argue that caution is advisable when trying to predict LUE by reflectance alone.

PSII recovery is largely dependent on temperature

The recovery of photosynthetic activity in spring, as indicated by the maximum quantum yield of PSII (F_v/F_m), is mainly driven by temperature (Fig. 25A). As a consequence, the recovery of ETR, NPQ and $1-q_P$ is also mainly temperature dependent

(Fig. 25B,C,D). We observed lower values of NPQ under conditions, where ETR is depressed, due to a sustained depression of F_m (Fig. 25B,C). This seasonal depression of F_m does not allow any recovery of the maximum fluorescence signal in the dark and thus limits the use of the fluorescence parameter NPQ. The calculation of NPQ relies on a dark-adapted control value of F_m in which the system is in a fully relaxed state. Under conditions of sustained quenching (e.g. in winter), when F_m is depressed and does not relax rapidly upon darkening of the leaves, the level of energy dissipation, calculated as NPQ, will be underestimated (Adams and Demmig-Adams, 2004). Under these conditions, excitation pressure ($1-q_P$) might be a better parameter to describe the imbalance of the energy that is absorbed and the energy that can be utilized (Fig. 25D). This observation is reflected in that the maximum quantum yield of PSII photochemistry, ETR as well as $1-q_P$ are all temperature dependent, whereas the differences in NPQ are temperature and light dependent (Fig. 25). The photosynthetic machinery of overwintering evergreens is well adapted to quickly resume photosynthesis once environmental conditions become favourable (Ottander and Öquist, 1991; Dolman *et al.*, 2002), which means an increase of ambient temperature to above zero degrees (Tanja *et al.*, 2003). Therefore, it is not surprising that the photosynthetic parameters measured in our experiment were affected to a larger extent by temperature than by light intensity (Fig. 25) (Blennow *et al.*, 1998; Lundmark *et al.*, 1998).

Effect of temperature and light on the xanthophyll cycle dynamics

In contrast, DEPS and the amount of zeaxanthin appeared to be both, temperature and light dependent (Fig. 26B,C). This reflects the fact that energy utilization is temperature dependent (Hüner *et al.*, 1998; Ensminger *et al.*, 2006), with higher temperatures resulting in higher photochemical quenching (ETR, Fig. 25F) and lower excitation pressure on PSII ($1-q_P$, Fig. 25H). Conversely, since the amount of ETR is equivalent, a higher light intensity at a given temperature causes an increased amount of excess light, which is reflected in higher $1-q_P$ (Fig. 25H) and NPQ values (Fig. 25G). Since zeaxanthin is an integral component of the excess energy quenching mechanisms through the xanthophyll cycle, this also entails a light dependency of zeaxanthin and

DEPS (Fig. 26B,C). Therefore, the amount of zeaxanthin, at best, can be used to estimate the amount of non-photochemical, but not necessarily photochemical quenching.

The PRI signal is affected by growth light intensity but not by temperature

PRI appears to be largely dependent on the light intensity rather than temperature (Fig. 27). Given the temperature dependence of the ETR, there was no strong correlation between PRI and ETR (Fig. 28). Under cold acclimated conditions, the ETR was suppressed considerably, while PRI was fairly variable. Upon transfer of the plants from winter to simulated spring conditions, the ETR recovered only slowly, without considerable changes in PRI (Fig. 28). An increase in PRI is only detectable 12 days after transfer, concomitant with an increase in ETR (Fig. 28).

During winter, the xanthophyll cycle is arrested in a photoprotective state where zeaxanthin cannot be reconverted into violaxanthin and thus the photochemical system is primed for sustained thermal dissipation of energy (Demmig-Adams and Adams, 2006). This sustained thermal dissipation occurs irrespective of the amount of excess light through the sustained structural changes in PSII (Demmig-Adams and Adams, 2006). The flexible component of NPQ is only regained once the deacclimation under warm temperatures has occurred (Ensminger *et al.*, 2004; Zarter *et al.*, 2006; Ensminger *et al.*, 2008). In addition, another possible reason why PRI cannot explain the variation in ETR might be that zeaxanthin-dependent quenching is only one of several pathways for non-photochemical quenching. It has been shown that in cold-acclimated *P. banksiana* (Busch *et al.*, 2007), as well as in overwintering evergreens and other plants (Horton *et al.*, 1991; Ruban *et al.*, 1993; Ottander *et al.*, 1995; Gilmore and Ball, 2000; Krol *et al.*, 2002), a major dissipation pathway is the aggregation of the light harvesting complex (LHCII). This pathway is zeaxanthin-independent and therefore is not reflected in changes in PRI. Another zeaxanthin-independent mechanism is PSII reaction center quenching in combination with high excitation pressure. In pine, reaction center quenching is maximal at low temperatures during the winter when the needles are photoinhibited (Ivanov *et al.*, 2002).

In conclusion, our study shows that the understanding of quenching mechanisms under different environmental conditions and in different seasons is crucial to understanding the PRI signal and how it reflects the LUE of forest canopies. The knowledge of how the physiological state of the plant influences energy quenching processes is indispensable in order to accurately model the carbon balance of the world's ecosystems. An increased understanding of the physiological processes underlying the ecosystem fluxes is necessary to move from a purely descriptive to a robust quantitative characterization of the annual global carbon cycle.

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Figures

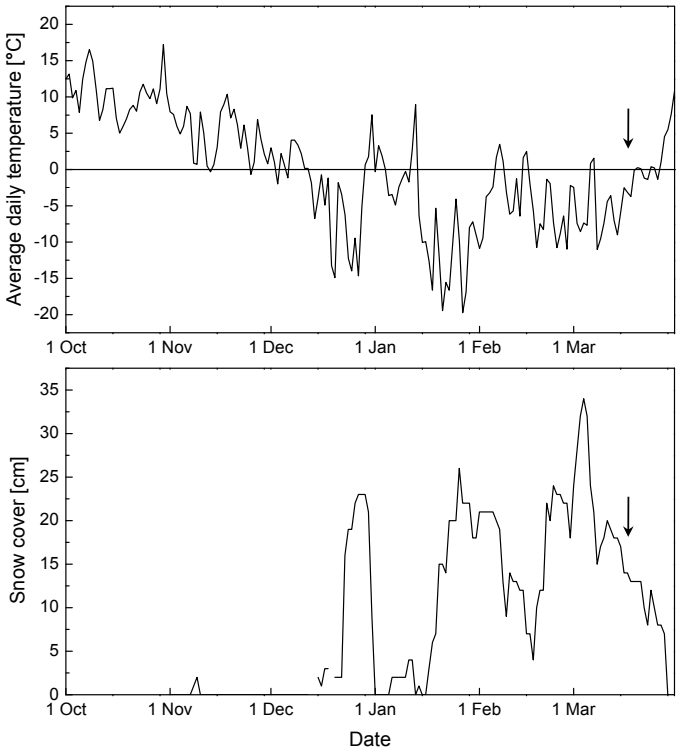
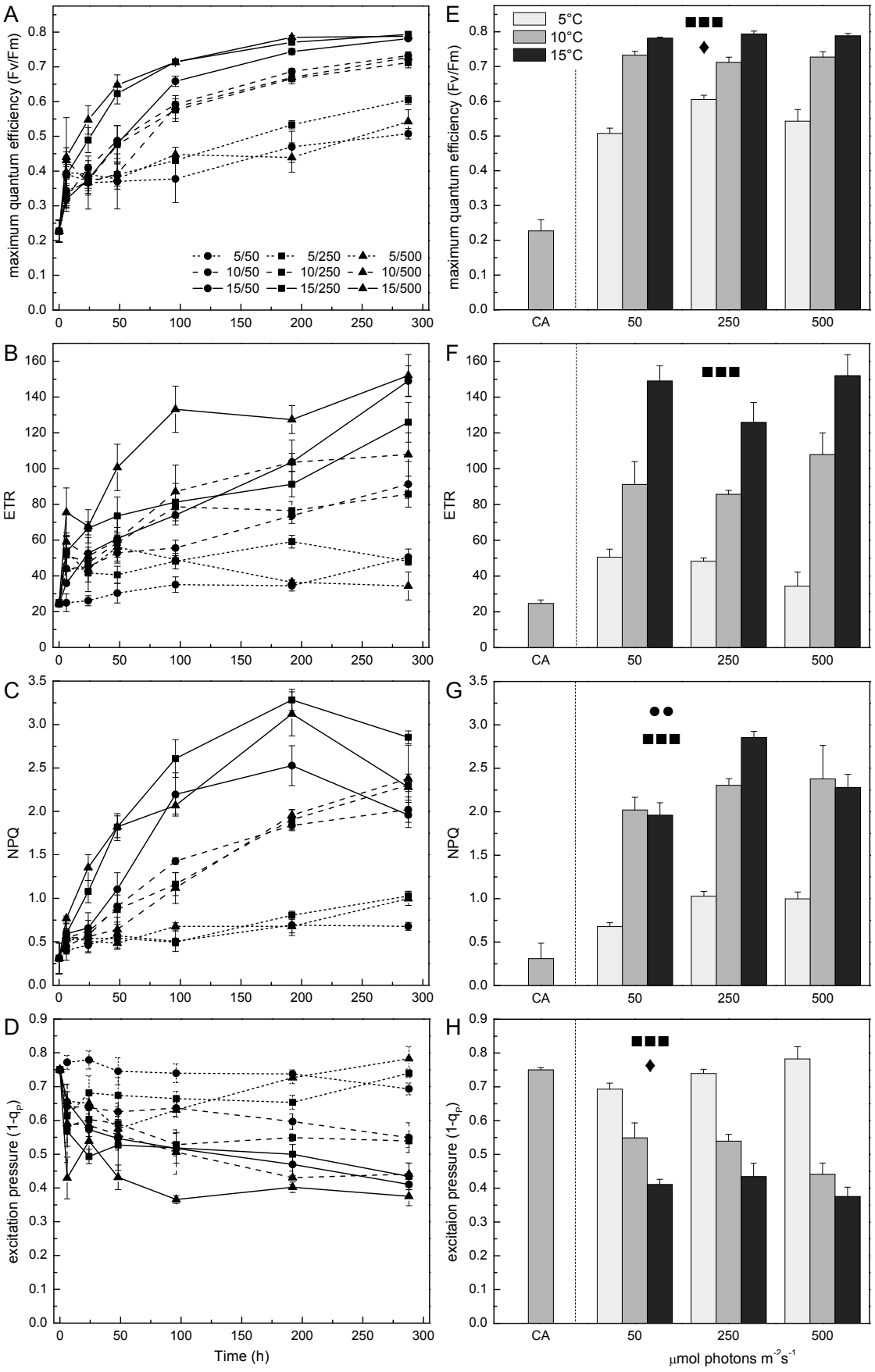


Figure 24: *Meteorological data of the time prior to transferring the plants to the experimental conditions on 18 March 2005. The arrow indicates the date of transfer. (A) Average daily temperature in °C; (B) Snow cover in cm.*

Figure 25: *(Opposite page) The effect of temperature and light intensity on chlorophyll fluorescence parameters in needles of* P. banksiana. *Recovery of (A) the maximum quantum efficiency (F_v/F_m); (B) the electron transport rate (ETR); (C) non-photochemical quenching (NPQ); (D) excitation pressure ($1-q_P$) before and 6 h, 12 h, 1, 2, 4, 8 and 12 days after the transfer to treatment conditions. Treatment conditions: dotted line: 5°C; dashed line: 10°C; solid line: 15°C;* ●, ■ *and* ▲*: 50, 250 and 500* μmol photons m^{-2} s^{-1}, *respectively. Values 12 days after the plants were transferred from winter to experimental conditions: (E) maximum quantum efficiency (F_v/F_m); (F) electron transport rate (ETR); (G) non-photochemical quenching (NPQ); (H) excitation pressure ($1-q_P$). CA represents the cold acclimated condition before the transfer.* ●, ■ *and* ◆ *indicate significant differences due to light intensity, temperature and their interactive effect, respectively. One symbol: $P < 0.05$; two symbols: $P < 0.01$; three symbols: $P < 0.001$. Each data point represents the average of n = 3 to 5 ± SE individual seedlings.*


A
maximum quantum efficiency (Fv/Fm)
5/50
5/250
5/500
10/50
10/250
10/500
15/50
15/250
15/500
B
ETR
C
NPQ
D
excitation pressure (1-qP)
Time (h)
E
5°C
10°C
15°C
maximum quantum efficiency (Fv/Fm)
CA
50
250
500
F
ETR
G
NPQ
H
excitaion pressure (1-qP)
µmol photons m-2s-1

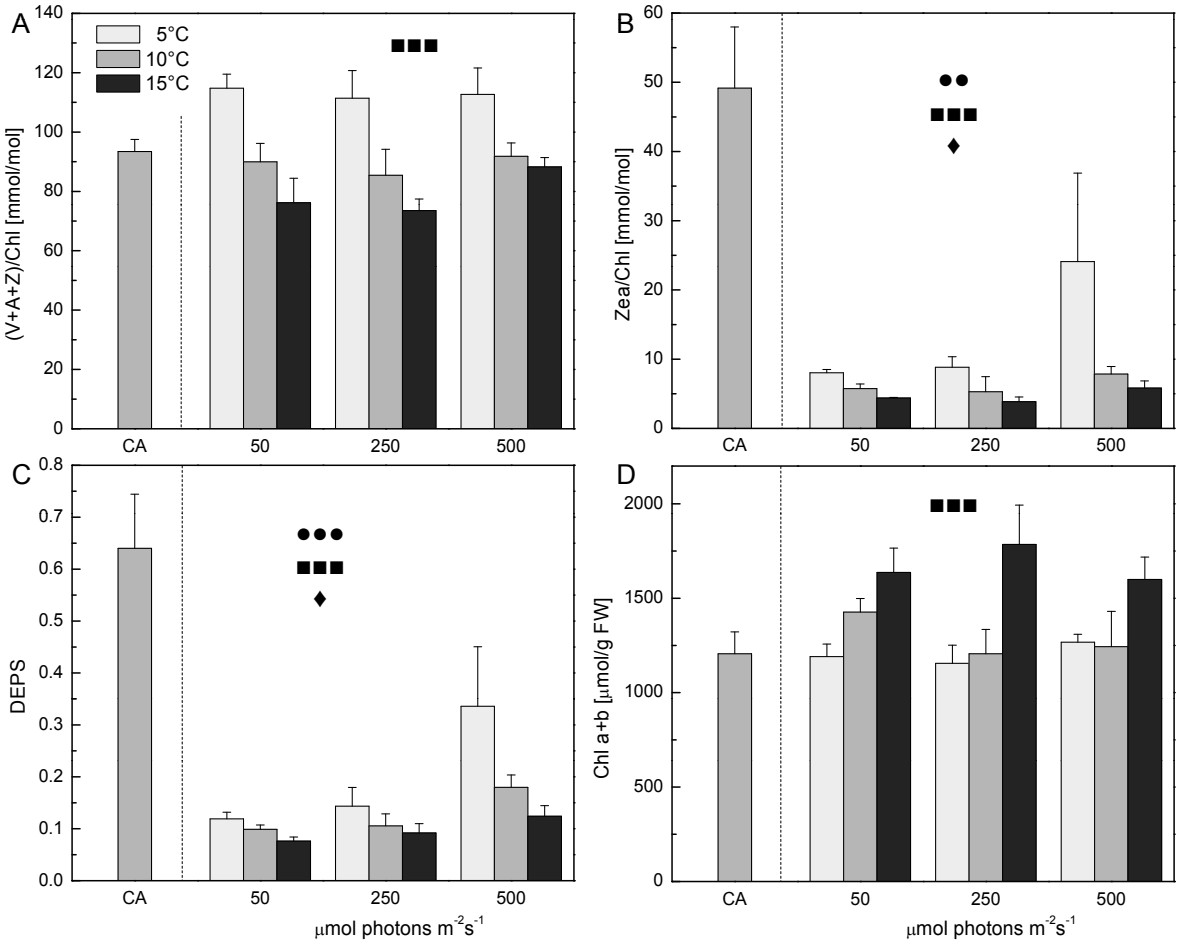


Figure 26: *The effect of temperature and light intensity on the composition of photosynthetic pigments in needles of* P. banksiana, *12 days after the plants were transferred from winter to experimental conditions. (A) total pool size of xanthophyll cycle pigments (V + A + Z) per total chlorophyll; (B) amount of zeaxanthin per total chlorophyll; (C) deepoxidation of the xanthophyll cycle pigments (DEPS), calculated as (0.5 A + Z)/(V + A + Z); (D) amount of chlorophyll a + b per fresh weight. CA represents the cold acclimated condition before the transfer. Each data point represents the average of n = 3 ± SE individual seedlings.* ●, ■ *and* ◆ *indicate significant differences due to light intensity, temperature and their interactive effect, respectively. One symbol: P < 0.05; two symbols: P < 0.01; three symbols: P < 0.001.*

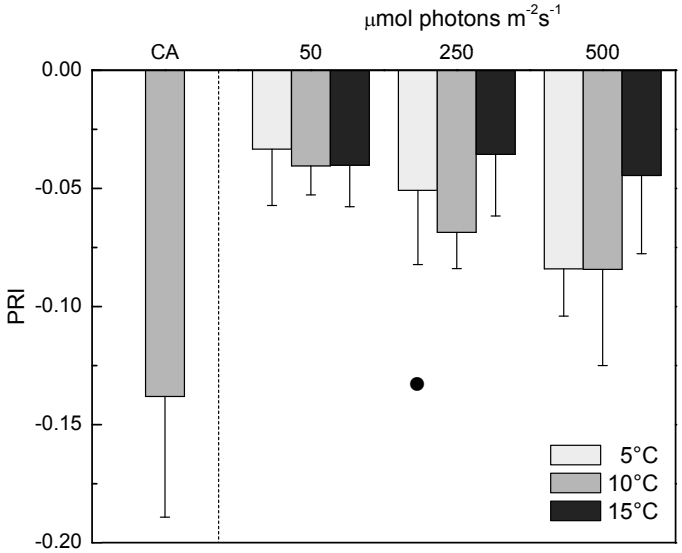


Figure 27: *The effect of temperature and light intensity on the photochemical reflectance index (PRI) in needles of* P. banksiana*, 12 days after the plants were transferred from winter to experimental conditions. CA represents the cold acclimated condition before the transfer. Each data point represents the average of n = 3 ±* SE *individual seedlings.* ●, ■ *and* ◆ *indicate significant differences due to light intensity, temperature and their interactive effect, respectively. One symbol: P < 0.05; two symbols: P < 0.01; three symbols: P < 0.001.*

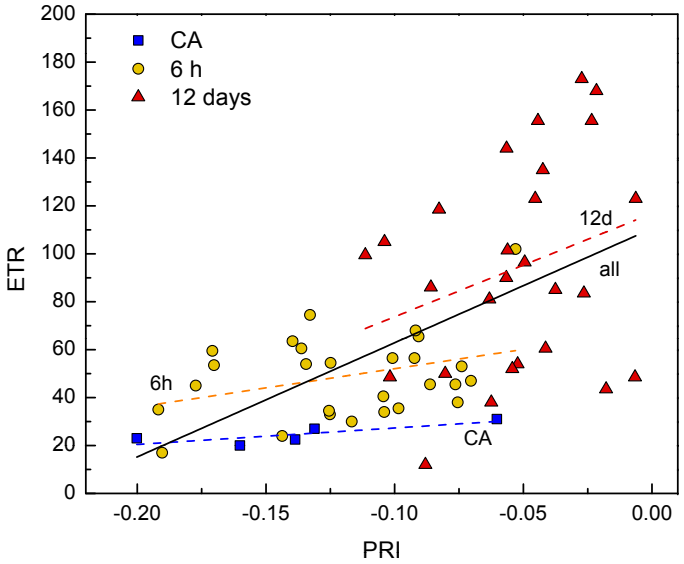


Figure 28: *Relationship between the electron transport rate (ETR) and photochemical reflectance index (PRI) in needles of* P. banksiana. *(□, blue) before the plants were transferred to experimental conditions (CA); (○, yellow) 6 hours after transfer (6h); (△, red) 12 days after transfer (12d). Adjusted R^2 for the correlations of different measurement times: $R^2_{CA} = 0.543$, $R^2_{6h} = 0.086$, $R^2_{12d} = 0.046$ and $R^2_{all} = 0.324$.*

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